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COMPLEXES OF ALPHA (2) MACROGLOBULIN AND ANTIGENIC MOLECULES FOR IMMUNOTHERAPY

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This application claims priority under 35 U.S.C. §120 to U.S. application no. 09/625,139 filed July 25, 2000, and claims benefit under 35 U.S.C. §119(e) of U.S. provisional patent application no. 60/209,266 filed June 2, 2000, both of which are incorporated by reference herein in their entirety.

This invention was made with government support under grant number CA64394 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to complexes of alpha (2) macroglobulin associated with antigenic molecules for use in immunotherapy. The invention relates to methods for using such compositions in the diagnosis and treatment of immune disorders, proliferative disorders, and infectious diseases.

2. BACKGROUND OF THE INVENTION

2.1. HEAT SHOCK PROTEINS

Heat shock proteins (HSPs), also referred to as stress proteins, were first identified as proteins synthesized by cells in response to heat shock. Hsps have classified into five families, based on molecular weight, Hsp100, Hsp90, Hsp70, Hsp60, and smHsp. Many members of these families were found subsequently to be induced in response to other stressful stimuli including nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens (see Welch, May 1993, Scientific American 56-64; Young, 1990, Annu. Rev. Immunol. 8:401-420; Craig, 1993, Science 260:1902-1903; Gething *et al.*, 1992, Nature 355:33-45; and Lindquist *et al.*, 1988, Annu. Rev. Genetics 22:631-677).

Heat shock proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from *E. coli* has about 50% amino acid sequence identity with Hsp70 proteins from excoriates (Bardwell *et al.*, 1984, Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families also show similarly high levels of intra-family conservation (Hickey *et al.*, 1989, Mol. Cell. Biol. 9:2615-2626; Jindal, 1989, Mol. Cell. Biol. 9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are

composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress.

Studies on the cellular response to heat shock and other physiological stresses revealed that the HSPs are involved not only in cellular protection against these adverse conditions, but also in essential biochemical and immunological processes in unstressed cells. Hsps accomplish different kinds of chaperoning functions. For example, members of the Hsp70 family, located in the cell cytoplasm, nucleus, mitochondria, or endoplasmic reticulum (Lindquist *et al.*, 1988, Ann. Rev. Genetics 22:631-677), are involved in the presentation of antigens to the cells of the immune system, and are also involved in the transfer, folding and assembly of proteins in normal cells. Hsps are capable of binding proteins or peptides, and releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

2.2. IMMUNOGENICITY OF HSP-PEPTIDE COMPLEXES

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (1988, Immunol. Today 9:78-83). In these studies, it was found that the molecules responsible for the individually distinct immunogenicity of these tumors were glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28:205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167:109-123). Further, Hsp70 was shown to elicit immunity to the tumor from which it was isolated but not to antigenically distinct tumors. However, Hsp70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides (Srivastava, 1993, Adv. Cancer Res. 62:153-177; Udono *et al.*, 1994, J. Immunol., 152:5398-5403; Suto *et al.*, 1995, Science 269:1585-1588).

Noncovalent complexes of HSPs and peptide, purified from cancer cells, can be used for the treatment and prevention of cancer and have been described in PCT publications WO 96/10411, dated April 11, 1996, and WO 97/10001, dated March 20, 1997 (U.S. Patent No. 5,750,119 issued April 12, 1998, and U.S. Patent No. 5,837,251 issued November 17, 1998,

respectively, each of which is incorporated by reference herein in its entirety). The isolation and purification of stress protein-antigen complexes has been described, for example, from pathogen-infected cells, and used for the treatment and prevention of infection caused by the pathogen, such as viruses, and other intracellular pathogens, including bacteria, protozoa, fungi and parasites (see, for example, PCT Publication WO 95/24923, dated September 21, 1995). Immunogenic stress protein-antigen complexes can also be prepared by in vitro complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of cancer and infectious diseases has been described in PCT publication WO 97/10000, dated March 20, 1997 (U.S. Patent No. 6,030,618 issued February 29, 2000. The use of stress protein-antigen complexes for sensitizing antigen presenting cells in vitro for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997 (see also U.S. Patent No. 5,985,270 issued November 16, 1999).

2.3. ALPHA (2) MACROGLOBULIN RECEPTOR

The α-macroglobulins are members of a protein superfamily of structurally related proteins which also comprises complement components C3, C4 and C5. The human plasma protein alpha (2) macroglobulin (α2M) is a 720 kDa homotetrameric protein primarily known as proteinase inhibitor and plasma and inflammatory fluid proteinase scavenger molecule (for review see Chu and Pizzo, 1994, Lab. Invest. 71:792). Alpha (2) macroglobulin is synthesized as a 1474 amino acid precursor, the first 23 of which function as a signal sequence that is cleaved to yield a 1451 amino acid mature protein (Kan *et al.*, 1985, Proc. Natl. Acad. Sci. U.S.A. 82:2282-2286).

Alpha (2) macroglobulin promiscuously binds to proteins and peptides with nucleophilic amino acid side chains in a covalent manner (Chu *et al.*, 1994, Ann. N.Y. Acad. Sci. 737:291-307) and targets them to cells which express the α2M receptor (α2MR) (Chu and Pizzo, 1993, J. Immunol. 150:48). Binding of α2M to the α2M receptor is mediated by the C-terminal portion of α2M (Holtet *et al.*, 1994, FEBS Lett. 344:242-246) and key residues have been identified (Nielsen et al., 1996, J. Biol. Chem. 271:12909-12912).

Generally known for inhibiting protease activity, α2M binds to a variety of proteases thorough multiple binding sites (*see, e.g.*, Hall *et al.*, 1981, Biochem. Biophys. Res. Commun. 100(1):8-16). Protease interaction with α2M results in a complex structural rearrangement called transformation, which is the result of a cleavage within the "bait" region of α2M after the proteinase becomes "trapped" by thioesters. The conformational change exposes residues required for receptor binding, allowing the α2M-proteinase complex to bind to the α2MR. Methylamine can induce similar conformational changes and cleavage as that induced by proteinases. The uncleaved form of α2M, which is not recognized by the

receptor, is often referred to as the "slow" form (s-α2M). The cleaved form is referred to as the "fast" form (f-α2M) (reviewed by Chu *et al.*, 1994, Ann. N.Y. Acad. Sci. 737:291-307).

Studies have shown that in addition to its proteinase-inhibitory functions, α2M, when complexed to antigens, can enhance the antigens' ability to be taken up by antigen presenting cells such as macrophages and presented to T cell hybridomas *in vitro* by up to two orders of magnitude (Chu and Pizzo, 1994, Lab. Invest. 71:792), and induce T cell proliferation (Osada et al., 1987, Biochem. Biophys. Res. Commun.146:26-31). Further evidence suggests that complexing antigen with α2M enhances antibody production by crude spleen cells *in vitro* (Osada *et al.*, 1988, Biochem. Biophys. Res. Commun. 150:883) elicits an *in vivo* antibody responses in experimental rabbits (Chu *et al.*, 1994, J. Immunol. 152:1538-1545) and mice (Mitsuda *et al.*, 1993, Biochem. Biophys. Res. Commun. 101:1326-1331). However, none of these studies have shown whether alpha2M-antigen complexes are capable of eliciting cytotoxic T cell responses *in vivo*.

2.4. IMMUNOGENICITY OF HEAT SHOCK/STRESS PROTEINS

Srivastava *et al.* demonstrated immune response to methylcholanthrene-induced sarcomas of inbred mice (1988, Immunol. Today 9:78-83). In these studies, it was found that the molecules responsible for the individually distinct immunogenicity of these tumors were identified as cell-surface glycoproteins of 96kDa (gp96) and intracellular proteins of 84 to 86kDa (Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3407-3411; Ullrich, S.J. *et al.*, 1986, Proc. Natl. Acad. Sci. USA 83:3121-3125). Immunization of mice with gp96 or p84/86 isolated from a particular tumor rendered the mice immune to that particular tumor, but not to antigenically distinct tumors. Isolation and characterization of genes encoding gp96 and p84/86 revealed significant homology between them, and showed that gp96 and p84/86 were, respectively, the endoplasmic reticular and cytosolic counterparts of the same heat shock proteins (Srivastava *et al.*, 1988, Immunogenetics 28:205-207; Srivastava *et al.*, 1991, Curr. Top. Microbiol. Immunol. 167:109-123). Further, Hsp70 was shown to elicit immunity to the tumor from which it was isolated but not to antigenically distinct tumors. However, Hsp70 depleted of peptides was found to lose its immunogenic activity (Udono and Srivastava, 1993, J. Exp. Med. 178:1391-1396). These observations suggested that the heat shock proteins are not immunogenic per se, but form noncovalent complexes with antigenic peptides, and the complexes can elicit specific immunity to the antigenic peptides (Srivastava, 1993, Adv. Cancer Res. 62:153-177; Udono *et al.*, 1994, J. Immunol., 152:5398-5403; Suto *et al.*, 1995, Science, 269:1585-1588).

The use of noncovalent complexes of stress proteins and peptides, purified from cancer cells, for the treatment and prevention of cancer, as well as the use of such complexes in combination with adoptive immunotherapy, has been described (see U.S. Patent No.

5,750,199; U.S. Patent No. 5,830,464; Patent Cooperation Treaty ("PCT") publications WO 96/10411, dated April 11, 1996; and WO 97/10001, dated March 20, 1997; each of which is incorporated by reference herein in its entirety. The purification of stress protein-peptide complexes from cell lysates has been described previously; stress protein-peptide complexes can be isolated from pathogen-infected cells and used for the treatment and prevention of infection caused by pathogens, such as viruses and other intracellular pathogens, including bacteria, protozoa, fungi and parasites (see PCT publication WO 95/24923, dated September 21, 1995).

Immunogenic stress protein-peptide complexes can also be prepared by *in vitro* complexing of stress protein and antigenic peptides, and the uses of such complexes for the treatment and prevention of infectious diseases and cancer has been described in PCT publication WO 97/10000, dated March 20, 1997. The use of heat shock proteins in combination with a defined antigen for the treatment of infectious diseases and cancer have also been described in PCT publication WO 97/06821, dated February 27, 1997. The administration of expressible polynucleotides encoding eukaryotic heat shock proteins to mammalian cells for stimulating an immune response, and for treatment of infectious diseases and cancer has been described in PCT publications, WO 97/06685 and WO 97/06828, both dated February 27, 1997. The use of stress protein-peptide complexes for sensitizing antigen presenting cells *in vitro* for use in adoptive immunotherapy is described in PCT publication WO 97/10002, dated March 20, 1997.

2.5. ANTIGEN PRESENTATION

Major histocompatibility complex (MHC) molecules present antigens on the cell surface of antigen-presenting cells. Cytotoxic T lymphocytes (CTLs) then recognize MHC molecules and their associated peptides and kill the target cell. Antigens are processed by two distinct antigen processing routes depending upon whether their origin is intracellular or extracellular. Intracellular or endogenous protein antigens, *i.e.*, antigens synthesized within the antigen-presenting cell, are presented by MHC class I (MHC I) molecules to CD8+ cytotoxic T lymphocytes. On the other hand, extracellular or exogenously synthesized antigenic determinants are presented on the cell surface of "specialized" or "professional" APCs (macrophages, for example) by MHC class II molecules to CD4+ T cells (see, generally, Fundamental Immunology, W.E. Paul (ed.), New York: Raven Press, 1984). This compartmental segregation of antigen processing routes is important to prevent tissue destruction that could otherwise occur during an immune response as a result of shedding of neighboring cell MHC I antigens.

The heat shock protein gp96 chaperones a wide array of peptides, depending upon the source from which gp96 is isolated (for review, see Srivastava *et al.*, 1998, Immunity 8: 657-

665). Tumor-derived gp96 carries tumor-antigenic peptides (Ishii *et al.*, 1999, J. Immunology 162:1303-1309); gp96 preparations from virus-infected cells carry viral epitopes (Suto and Srivastava, 1995, Science 269:1585-1588; Nieland *et al.*, 1998, Proc. Natl. Acad. Sci. USA 95:1800-1805), and gp96 preparations from cells transfected with model antigens such as ovalbumin or β-galactosidase are associated with the corresponding epitopes (Arnold *et al.*, 1995, J. Exp. Med.182:885-889; Breloer *et al.*, 1998, Eur. J. Immunol. 28:1016-1021). The association of gp96 with peptides occurs in vivo (Menoret and Srivastava, 1999, Biochem. Biophys. Research Commun. 262:813-818). Gp96-peptide complexes, whether isolated from cells (Tamura *et al.*, 1997, Science 278:117-120), or reconstituted *in vitro* (Blachere *et al.*, 1997, J. Exp. Med. 186:1183-1406) are excellent immunogens and have been used extensively to elicit CD8+ T cell responses specific for the gp96-chaperoned antigenic peptides.

The capacity of gp96-peptide complexes to elicit an immune response is dependent upon the transfer of the peptide to MHC class I molecules of antigen-presenting cells (Suto and Srivastava, 1995, *supra*). Endogenously synthesized antigens chaperoned by gp96 in the endoplasmic reticulum [ER] can prime antigen-specific CD8+ T cells (or MHC I-restricted CTLs) *in vivo*; this priming of CD8+ T cells requires macrophages. However, the process whereby exogenously introduced gp96-peptide complexes elicit the antigen-specific CD8+ T cell response is not completely understood since there is no established pathway for the translocation of extracellular antigens into the class I presentation machinery. Yet antigenic peptides of extracellular origin associated with HSPs are somehow salvaged by macrophages, channeled into the endogenous pathway, and presented by MHC I molecules to be recognized by CD8+ lymphocytes (Suto and Srivastava, 1995, *supra*; Blachere *et al.*, 1997, J. Exp. Med. 186:1315-22).

Several models have been proposed to explain the delivery of extracellular peptides for antigen presentation. One proposal, known as the "direct transfer" model, suggests that HSP-chaperoned peptides are transferred to MHC I molecules on the cell surface of macrophages for presentation to CD8+ T lymphocytes. Another suggestion is that soluble extracellular proteins can be trafficked to the cytosol via constitutive macropinocytosis in bone marrow-derived macrophages and dendritic cells (Norbury *et al.*, 1997, Eur. J. Immunol. 27:280-288). Yet another proposed mechanism is that HSPs are taken up by the MHC class I molecules of the macrophage, which stimulate the appropriate T cells (Srivastava *et al.*, 1994, Immunogenetics 39:93-98. Others have suggested that a novel intracellular trafficking pathway may be involved for the transport of peptides from the extracellular medium into the lumen of ER (Day *et al.*, 1997, Proc. Natl. Acad. Sci. 94:8064-8069; Nicchitta, 1998, Curr. Opin. in Immunol. 10:103-109). Further suggestions include the involvement of phagocytes which (a) possess an ill-defined pathway to shunt protein

from the phagosome into the cytosol where it would enter the normal class I pathway; (b) digest ingested material in lysosomes and regurgitate peptides for loading on the surface to class I molecules (Bevan, 1995, J. Exp. Med. 182:639-41).

Still others have proposed a receptor-mediated pathway for the delivery of extracellular peptides to the cell surface of APS for antigen presentation. In view of the extremely small quantity of gp96-chaperoned antigenic peptides required for immunization (Blachere *et al.*, 1997, *supra*), and the strict dependence of immunogenicity of gp96-peptide complexes on functional antigen presenting cells (APCs) (Udono *et al.*, 1994, Proc. Natl. Acad. Sci. U.S.A. 91:3077-3081), APCs had been proposed to possess receptors for gp96 (Srivastava *et al.*, 1994, Immunogenetics 39:93-98). Preliminary microscopic evidence consistent with such receptors has been recently obtained (Binder *et al.*, 1998, Cell Stress & Chaperones 3 (Supp.1):2.; Arnold-Schild *et al.*, 1999, J. Immunol. 162: 3757-3760; and Wassenberg *et al.*, 1999, J. Cell Sci. 1:12). One hypothesis is that the mannose receptor is used in the uptake of gp96, but no mechanism has been proposed for the non-glycosylated HSPs, such as Hsp70 (Ciupitu *et al.*, 1998, J. Exp. Med., 187:685-691).

The identification and characterization of specific molecules involved in HSP-mediated antigen presentation of peptides, could provide useful reagents and techniques for eliciting specific immunity by HSP and HSP-peptide complexes, and for developing novel diagnostic and therapeutic methods.

Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention provides complexes comprising alpha (2) macroglobulin ("α2M") and methods for their use in immunotherapy. The invention is based, in part, on the Applicant's discovery that α2M directly competes for the binding of heat shock protein gp96 to the α2M receptor, indicating that α2M and HSPs may bind to a common recognition site on the alpha (2) macroglobulin receptor. Thus, because HSPs and α2M have a number of common functional attributes, such as the ability to bind peptides and the recognition and uptake by the alpha (2) macroglobulin receptor, the Applicants have discovered that α2M can be used in the methods described herein for immunotherapy against cancer and infectious disease. Alpha-2-macroglobulin can form complexes with antigens, which are taken up by antigen presenting cells ("APCs") via the alpha (2) macroglobulin receptor, also known as LDL (low-density lipoprotein) Receptor-Related Protein ("LRP") or CD91. Thus, the invention provides methods and compositions for using specific α2M-antigenic molecule

complexes for targeting an immune response against immune disorders, proliferative disorders, and infectious diseases.

The invention encompasses complexes of alpha (2) macroglobulin noncovalently associated antigenic molecules, recombinant cells that express the complexes of α2M associated with antigenic molecules, and antibodies and other molecules that specifically recognize α2M-antigenic molecule complexes. The invention also provides methods for using these compositions in the diagnosis and treatment of immune disorders, proliferative disorders, and infectious diseases.

As used herein, an alpha (2) macroglobulin is associated with an antigenic molecule is bound to the antigenic molecule by a covalent or noncovalent bond. A covalent bond can be a peptide bond or a thioester linkage, for example. Thus, fusion proteins between alpha (2) macroglobulin and an antigenic molecule are within the scope of the invention.

The invention provides a pharmaceutical composition comprising an amount of a molecular complex effective for treatment or prevention of an infectious disease or cancer, and a pharmaceutically acceptable carrier, said molecular complex comprising an alpha (2) macroglobulin polypeptide noncovalently associated with an antigenic molecule which displays the antigenicity of an antigen of an infectious agent or an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type. As used herein a cell type of a cancer cell, refers to the cell type of the tissue of origin, *e.g.*, breast, lung, ovarian. In one embodiment, the antigenic molecule displays the antigenicity of an antigen of an infectious agent. In another embodiment, the antigenic molecule displays the antigenicity of an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type. In another embodiment, the antigenic molecule is a tumor specific antigen or a tumor-associated antigen. In another embodiment, the antigenic molecule displays the antigenicity of an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type.

In another embodiment, the molecular complex effective for treatment or prevention of an infectious disease or cancer, comprising the alpha (2) macroglobulin polypeptide noncovalently associated with the antigenic molecule is purified. In particular, the term "purified" molecular complexes refer to complexes which are at least 65% 75%, 80%, 85%, 90%, 95%, 98% or 100% noncovalent complexes of the alpha (2) macroglobulin polypeptide and the antigenic molecule. In another embodiment, the purified molecular complex comprising an alpha (2) macroglobulin polypeptide associated with an antigenic molecule of an infectious agent or an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type.

The invention further provides a purified population of molecular complexes in which at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 100% of the complexes

comprise an alpha (2) macroglobulin noncovalently associated with an antigenic molecule. Also provided by the invention is a purified population of molecular complexes purified from a recombinant cell in which at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 100% of the complexes comprise an alpha (2) macroglobulin noncovalently associated with an antigenic molecule.

The invention also provides a recombinant cell infected with a pathogen and transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin polypeptide, which alpha (2) macroglobulin polypeptide associates with an antigenic molecule, when said antigenic molecule is present, to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. The invention provides a recombinant cancer cell transformed with a nucleic acid comprising a nucleotide sequence that (i) is operably linked to a promoter, and (ii) encodes an alpha (2) macroglobulin polypeptide, which alpha (2) macroglobulin polypeptide associates with an antigenic molecule, when said antigen is present, to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. In another embodiment, the invention provides a recombinant cell transformed with (i) a first nucleic acid comprising a first nucleotide sequence that is operably linked to a first promoter and encodes an alpha (2) macroglobulin polypeptide, and (ii) a second nucleic acid comprising a second nucleotide sequence that is operably linked to a second promoter and encodes an antigenic molecule, such that the alpha (2) macroglobulin polypeptide and the antigenic molecule are expressed within the cell and associate with each other to form a complex that in sufficient amount is capable of eliciting an immune response to the antigenic molecule. In various embodiments, the recombinant cells are human cells. In various embodiments, the pharmaceutical composition comprises a recombinant cell and a pharmaceutically acceptable carrier.

In one embodiment, a method is provided for preparing a complex of an alpha (2) macroglobulin polypeptide noncovalently associated with an antigenic molecule, said alpha (2) macroglobulin polypeptide comprising: (a) culturing a cell transformed with a nucleic acid comprising a nucleotide sequence encoding the alpha (2) macroglobulin polypeptide, under conditions such that said alpha (2) macroglobulin polypeptide is expressed by the cells and associates with an antigenic molecule of the cell; and (b) recovering a population of complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with antigenic molecules from the cells.

The invention further provides a method for preparing an alpha (2) macroglobulin polypeptide noncovalently associated with antigenic molecules derived from one or more antigens of an infectious agent, comprising: culturing infected cells, transformed with a nucleic acid comprising a nucleotide sequence encoding the alpha (2) macroglobulin

polypeptide, or fragment, analog, or variant thereof, and operably linked to a promoter, under conditions such that the alpha (2) macroglobulin polypeptideis expressed by the cells and associates with peptides of the cells; and (b) recovering from the cells a population of complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with peptides derived from the infectious agent. In one embodiment, the method further comprises purifying the complexes. In another embodiment, the method further comprising purifying the complexes by affinity chromatography.

The invention further provides a method of treating or preventing an infectious disease in an individual having an infectious disease comprising administering to the individual one or more immunogenic complexes of an alpha (2) macroglobulin polypeptide noncovalently associated with a first antigenic molecule, wherein the first antigenic molecule displays the antigenicity of an antigen of an infectious agent of the infectious disease. In another embodiment, the method further comprises, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of alpha (2) macroglobulin polypeptide noncovalently associated with a second antigenic molecule, said second antigenic molecule displaying the antigenicity of a second antigenic molecule of said infectious agent.

The invention further provides a method of treating or preventing an infectious disease in a subject having an infectious disease comprising: a) culturing an infected cell transformed with a nucleic acid comprising a nucleotide sequence encoding an alpha (2) macroglobulin polypeptide, said infected cell displaying the antigenicity of an antigen of an infectious agent of the infectious disease, said nucleotide sequence being operably linked to a promoter, under conditions such that the alpha (2) macroglobulin polypeptide is expressed by the infected cells and associates with antigenic molecules of the cell; b) recovering complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with antigenic molecules from the infected cell; and c) administering to the subject an amount of the recovered complexes effective to treat or prevent the infectious disease. In one embodiment, the method further comprises, prior to step (a), the step of obtaining infected cells from the subject and transforming the infected cells with the nucleic acid. In another embodiment, the method further comprises, prior to step (a), the step of obtaining the infected cell from one or more individuals and transforming the infected cells with the nucleic acid, said one or more individuals being different from the subject and having the same type of infectious disease as the subject.

The invention further provides a method of treating or preventing an infectious disease in a subject having an infectious disease comprising: a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding an alpha (2) macroglobulin polypeptide and

(ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen of an infectious agent of the infectious disease; b) recovering complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with the antigen; and c) administering to the subject an amount of the recovered complexes effective to treat or prevent the infectious disease. In various embodiments, the infectious disease is caused by an infectious agent selected from the group consisting of a virus, a bacterium, a fungus, and a parasite.

Also provide by the invention is a method of treating or preventing cancer in an individual having a type of cancer or in whom prevention of a type of cancer is desired comprising administering to the individual an immunogenic complex of an alpha (2) macroglobulin polypeptide noncovalently associated with a first antigenic molecule, wherein either (a) the first antigenic molecule displays antigenicity of said type of cancer or a metastasis thereof; or (b) the complex is obtained by recovering complexes from said type of cancer cells or a metastasis thereof that recombinantly express the alpha (2) macroglobulin polypeptide. In one embodiment, this method further comprises, before, concurrently or after administration of the immunogenic complex, administering to the individual a composition comprising antigen presenting cells sensitized *in vitro* with a sensitizing amount of a second complex of an alpha (2) macroglobulin noncovalently associated with a second antigenic molecule, said second antigenic molecule displaying the antigenicity of an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type. an infectious agent of the infectious disease. In another embodiment, the first antigenic molecule is an antigen overexpressed in a cancer cell relative to its expression in a noncancerous cell of said cell type.

In one embodiment, a method is provided for treating or preventing cancer in a subject having a type of cancer or in whom prevention of a type of cancer is desired comprising: a) culturing a cancer cell of said type transformed with a nucleic acid comprising a nucleotide sequence encoding an alpha (2) macroglobulin polypeptide said nucleotide sequence being operably linked to a promoter, under conditions such that the alpha (2) macroglobulin polypeptide is expressed by the cancer cell and associates with at least one antigenic molecule of the cell; b) recovering complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with at least one antigenic molecule from the cancer cell; and c) administering to the subject an amount of the recovered complexes effective to treat or prevent cancer. In one embodiment, the method further comprises, prior to step (a), the step of obtaining cancer cells from the subject and transforming the cancer cells with the nucleic acid. In another embodiment, the method further comprises, prior to step (a), the step of obtaining cancer cells from one or more individuals and transforming the cancer cells with

the nucleic acid, said one or more individuals being different from the subject and having the same type of cancer as the subject.

The invention further provides a method of treating or preventing cancer in a subject having a type of cancer or in whom prevention of a type of cancer is desired comprising: a) culturing a recombinant cell transformed with (i) a first nucleic acid encoding an alpha (2) macroglobulin polypeptide, and (ii) a second nucleic acid encoding an antigenic molecule displaying the antigenicity of an antigen of a cancer cell; b) recovering complexes of the alpha (2) macroglobulin polypeptide noncovalently associated with the antigen; and c) administering to the subject an amount of the recovered complexes effective to treat or prevent cancer.

As used herein a "type of cancer" refers to *e.g.*, melanoma, breast cancer, renal carcinoma, or a metastasis thereof, where a metastasis refers to the same type of cancer as the cell of origin. In various embodiments, the cancer is selected from the group consisting of fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemia, lymphoma, multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

The invention also encompasses a method for treating an autoimmune disorder comprising administering to a mammal in need of such treatment an antibody specific for alpha (2) macroglobulin. In one embodiment, the antibody is purified.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A-D . Identification of an 80 kDa polypeptide as a putative gp96 receptor. **A.** Confocal microscopy of re-presentation-competent RAW264.7 cells stained with gp96-FITC and **B.** with albumin-FITC. **C.** SDS-PAGE analysis of detergent extracts of plasma membranes from surface biotinylated RAW264.7 (re-presentation-competent) or P815 cells

(representation-incompetent) eluted from gp96 or albumin-Sepharose (SA) columns and stained with silver stain (top) or avidin-peroxidase (bottom). **D.** gp96-SASD-I^{125} was cross-linked to live peritoneal macrophages (MO) or P815 cells, and the cell lysates examined by SDS-PAGE and autoradiography. Various components were omitted as controls, as indicated.

FIG. 2A-B. Anti-p80 antiserum detects an 80 kDa molecule and inhibits re-presentation of gp96-chaperoned AHl peptide by macrophage. **A.** Pre-immune and immune sera were used to probe blots of plasma membrane extracts of RAW264.7, peritoneal macrophages (both cell types re-presentation-competent), or P815 cells. **B.** Re-presentation of gp96-chaperoned peptide AHI. Sera were added at the final dilution indicated. The solid cross indicates the level of T cell stimulation when the APCs were pulsed directly with the AHi peptide. The open cross indicates the corresponding value with unpulsed APCs.

FIG. 3A-C. Protein microsequencing of the 80 kDa protein. **A.** Analysis of a single tryptic (GALHIYHQR) peptide by tandem- mass spectrometry. All possible b- and y-ion series together with identified b-ion series (red) and y-ion series (blue) are shown. **B.** Collision-induced dissociation (CID) spectrum of this peptide is shown. **C.** Four identified peptides from the α2M receptor, peptide mass, and sequence are shown.

FIG. 4. α2-Macroglobulin inhibits re-presentation of gp96-chaperoned AH1 peptide by macrophage. The solid cross indicates the level of T cell stimulation when the APCs were pulsed directly with the AH1 peptide. The open cross indicates the corresponding value with unpulsed APCs.

FIG. 5. α2M receptor is a sensor of necrotic cell death due to its ability to detect extracellular gp96. Conversely, receptors (psR) for phosphatidyl serine (ps) detect apoptotic cell death.

FIG. 6A-B. A. The mouse α2MR cDNA (SEQ ID NO:1) and predicted open reading frame of murine α2MR protein (Genbank accession no. CAA47817). **B.** The murine α2M protein (SEQ ID NO:2), with residues identified by microsequencing an 80 kDa, gp96-interacting fragment of the receptor highlighted in bold.

FIG. 7A-B. A. Amino acid sequence of α2M (SEQ ID NO: 3). **B.** Nucleotide sequence of α2M (SEQ ID NO: 4). The 138 amino acid sequence (SEQ ID NO.: 5) of the receptor binding domain from α2M is underlined.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for alpha (2) macroglobulin ("α2M") vaccines for use in immunotherapy. The invention is based, in part, on the Applicant's discovery that α2M blocks uptake of heat shock proteins by antigen presenting cells. In particular, the invention provides complexes of α2M associated with antigenic molecules, which are recognized by the alpha (2) macroglobulin receptor on antigen presenting cells ("APCs"), and are presented by such cells to the immune system. Thus, the invention provides methods and compositions for using specific α2M-antigenic molecule complexes for targeting an immune response against immune disorders, proliferative disorders, and infectious diseases.

The human plasma protein alpha (2) macroglobulin is a 720 kDa homotetrameric proteinase inhibitor primarily known as proteinase inhibitor and plasma and inflammatory fluid proteinase scavenger molecule (for review see Chu and Pizzo, 1994, Lab. Invest. 71:792). During proteolytic activation of α2M, non-proteolytic ligands can become incorporated, covalently and noncovalently, to the activated thioesters (see Osada *et al.*, 1987, Biochem. Biophys. Res. Comm. 146:26-31; Osada *et al.*, 1988, Biochem. Biophys. Res. Comm. 150:883-889; Chu and Pizzo, 1993, J. Immunology 150: 48-58; Chu *et al.*, 1994, 152:1538-1545; Mitsuda *et al.*, 1993, Biochem. Biophys. Res. Comm. 191:1326-1331). As described herein, when complexes formed between α2M and an antigenic molecule having the antigenicity of a cancer cell antigen or of a pathogen, such α2M –antigenic molecule complexes can be used to stimulate a cytotoxic T cell response directed against the α2M incorporated antigen. Such complexes can be used as immunotherapeutic agents to treat cancer and infectious diseases.

Described in detailed hereinbelow are methods and compositions for use in preparation and delivery of such therapeutic α2M–antigenic molecule complexes. The invention encompasses complexes of alpha (2) macroglobulin associated antigenic molecules, antigenic cells that express the α2M, and antibodies and other molecules that specifically recognize α2M-antigenic molecule complexes. The invention also relates to methods for using these compositions in the diagnosis and treatment of immune disorders, proliferative disorders, and infectious diseases.

5.1 COMPOSITIONS OF THE INVENTION

The present invention provides compositions that can be used in immunotherapy against proliferative disorders, infectious diseases, and immune disorders. Such compositions include antibodies that specifically recognize α2M complexes, isolated antigenic cells that express α2M complexes, and recombinant cells that contain recombinant α2M and sequences encoding antigenic molecules.

It is contemplated that the definition of α2M, as used herein, embraces other polypeptide fragments, analogs, and variants of α2M having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with α2M, and is capable of forming a complex with an antigenic molecule, which complex is capable of being taken up by an antigen presenting cell and eliciting an immune response against the antigenic molecule. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al., 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res.25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul *et al.*, 1997, *supra*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see http://www.ncbi.nlm.nih.gov). Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The immunogenic α2M-antigenic molecule complexes of the invention may include any complex containing an α2M and an antigenic peptide that is capable of inducing an immune response in a mammal.

α2M and/or antigenic molecules can be purified from natural sources, chemically synthesized, or recombinantly produced.

5.1.1. α2M POLYPEPTIDES

The alpha (2) macroglobulin complex of the invention is comprised of an alpha (2) macroglobulin polypeptide associated with an antigenic peptide. Alpha (2) macroglobulin polypeptides may be produced by recombinant DNA techniques, synthetic methods, or by enzymatic or chemical cleavage of native α2M polypeptides. Described herein are methods for producing such α2M polypeptides..

5.1.1.1 ISOLATION OF α2M GENE SEQUENCES

In various aspects, the invention relates to compositions comprising amino acid sequences of α2M, and fragments, derivatives, analogs, and variants thereof. Nucleic acids encoding α2M are provided, as well as nucleic acids complementary to and capable of hybridizing to such nucleic acids.

Any eukaryotic cell may serve as the nucleic acid source for obtaining the coding region of an α2M gene. Nucleic acid sequences encoding α2M can be isolated from vertebrate, mammalian, as well as primate sources, including humans.

Amino acid sequences and nucleotide sequences of naturally occurring α2M polypeptides are generally available in sequence databases, such as GenBank. Non-limiting examples of α2M sequences that can be used for preparation of the α2M polypeptides of the invention are as follows: Genbank Accession Nos. M11313, P01023, AAA51551; Kan *et al.*, 1985, Proc. Nat. Acad. Sci. 82: 2282-2286. Due to the degeneracy of the genetic code, the term "α2M gene", as used herein, refers not only to the naturally occurring nucleotide sequence but also encompasses all the other degenerate DNA sequences that encode an α2M polypeptide. Computer programs, such as Entrez, can be used to browse the database, and retrieve any amino acid sequence and genetic sequence data of interest by accession number. These databases can also be searched to identify sequences with various degrees of similarities to a query sequence using programs, such as FASTA and BLAST, which rank the similar sequences by alignment scores and statistics. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res.25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (Altschul

et al., 1997, *supra*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (see http://www.ncbi.nlm.nih.gov).

The DNA may be obtained by standard procedures known in the art by DNA amplification or molecular cloning directly from a tissue, cell culture, or cloned DNA (*e.g.*, a DNA "library"). Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the α2M gene should be cloned into a suitable vector for propagation of the gene.

In a preferred embodiment, DNA can be amplified from genomic or cDNA by polymerase chain reaction (PCR) amplification using primers designed from the known sequence of a related or homologous α2M. PCR is used to amplify the desired sequence in DNA clone or a genomic or cDNA library, prior to selection. PCR can be carried out, *e.g.*, by use of a thermal cycler and Taq polymerase (sold under the trademark GENE AMP). The DNA being amplified can include cDNA or genomic DNA from any species. Oligonucleotide primers representing known nucleic acid sequences of related HSPs can be used as primers in PCR. In a preferred aspect, the oligonucleotide primers represent at least part of the α2M gene that is highly conserved between α2M genes of different species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known α2M nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization, moderately stringent conditions are preferred. After successful amplification, the sequence encoding an α2M may be cloned and sequenced. If the size of the coding region of the α2M gene being amplified is too large to be amplified in a single PCR, several PCR covering the entire gene, preferably with overlapping regions, may be carried out, and the products of the PCR ligated together to form the entire coding sequence. Alternatively, if a segment of an α2M gene is amplified, that segment may be cloned, and utilized as a probe to isolate a complete cDNA or genomic clone.

In another embodiment, for the molecular cloning of an α2M gene from genomic DNA, DNA fragments are generated to form a genomic library. Since some of the sequences encoding related α2Ms are available and can be purified and labeled, the cloned DNA fragments in the genomic DNA library may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction

enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available.

Alternatives to isolating the α2M genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes α2M. For example, RNA for cDNA cloning of the α2M gene can be isolated from cells which express α2M. A cDNA library may be generated by methods known in the art and screened by methods, such as those disclosed for screening a genomic DNA library. If an antibody to α2M is available, α2M may be identified by binding of labeled antibody to the putatively α2M synthesizing clones.

Other specific embodiments for the cloning of a nucleotide sequence encoding an α2M, are presented as examples but not by way of limitation, as follows:

In a specific embodiment, nucleotide sequences encoding α2M proteins within a family can be identified and obtained by hybridization with a probe comprising nucleotide sequence encoding α2M under conditions of low to medium stringency.

By way of example and not limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792). Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations).

An α2M gene fragment can be inserted into an appropriate cloning vector and introduced into host cells so that many copies of the gene sequence are generated. A large number of vector-host systems known in the art may be used such as, but not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene).

Any technique for mutagenesis known in the art can be used to modify individual nucleotides in a DNA sequence, for purpose of making amino acid substitution(s) in the expressed peptide sequence, or for creating/deleting restriction sites to facilitate further manipulations. Such techniques include but are not limited to, chemical mutagenesis, *in*

vitro site-directed mutagenesis (Hutchinson *et al.*, 1978, J. Biol. Chem 253:6551), oligonucleotide-directed mutagenesis (Smith, 1985, Ann. Rev. Genet. 19:423-463; Hill *et al.*, 1987, Methods Enzymol. 155:558-568), PCR-based overlap extension (Ho *et al.*, 1989, Gene 77:51-59), PCR-based megaprimer mutagenesis (Sarkar *et al.*, 1990, Biotechniques, 8:404-407), *etc.* Modifications can be confirmed by double stranded dideoxy DNA sequencing.

The polymerase chain reaction (PCR) is commonly used for obtaining genes or gene fragments of interest. For example, a nucleotide sequence encoding α2M polypeptide of any desired length can be generated using PCR primers that flank the nucleotide sequence encoding α2M, or the peptide-binding domain thereof. Alternatively, an α2M gene sequence can be cleaved at appropriate sites with restriction endonuclease(s) if such sites are available, releasing a fragment of DNA encoding α2M, or the peptide-binding domain thereof. If convenient restriction sites are not available, they may be created in the appropriate positions by site-directed mutagenesis and/or DNA amplification methods known in the art (see, for example, Shankarappa *et al.*, 1992, PCR Method Appl. 1:277-278). The DNA fragment that encodes α2M, or the peptide-binding domain thereof, is then isolated, and ligated into an appropriate expression vector, care being taken to ensure that the proper translation reading frame is maintained.

Alpha (2) macroglobulin polypeptides of the invention may be expressed as fusion proteins to facilitate recovery and purification from the cells in which they are expressed. For example, an α2M polypeptide may contain a signal sequence leader peptide to direct its translocation across the ER membrane for secretion into culture medium. Further, an α2M polypeptide may contain an affinity label, such as a affinity label, fused to any portion of the α2M polypeptide not involved in binding antigenic peptide, such as for example, the carboxyl terminal. The affinity label can be used to facilitate purification of the protein, by binding to an affinity partner molecule.

Various methods for production of such fusion proteins are well known in the art. The manipulations which result in their production can occur at the gene or protein level, preferably at the gene level. For example, the cloned coding region of an α2M polypeptide may be modified by any of numerous recombinant DNA methods known in the art (Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Ausubel *et al.*, in Chapter 8 of Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York). It will be apparent from the following discussion that substitutions, deletions, insertions, or any combination thereof are introduced or combined to arrive at a final nucleotide sequence encoding an α2M polypeptide.

In various embodiments, fusion proteins comprising the α2M polypeptide may be made using recombinant DNA techniques. For example, a recombinant gene encoding an

α2M polypeptide may be constructed by introducing an α2M gene fragment in the proper reading frame into a vector containing the sequence of an affinity label, such that the α2M polypeptide is expressed as a peptide-tagged fusion protein. Affinity labels, which may be recognized by specific binding partners, may be used for affinity purification of the α2M polypeptide.

In a preferred embodiment, the affinity label is fused at its amino terminal to the carboxyl terminal of α2M. The precise site at which the fusion is made in the carboxyl terminal is not critical. The optimal site can be determined by routine experimentation.

A variety of affinity labels known in the art may be used, such as, but not limited to, the immunoglobulin constant regions, polyhistidine sequence (Petty, 1996, Metal-chelate affinity chromatography, in Current Protocols in Molecular Biology, Vol. 2, Ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience), glutathione S-transferase (GST; Smith, 1993, Methods Mol. Cell Bio. 4:220-229), the *E. coli* maltose binding protein (Guan *et al.*, 1987, Gene 67:21-30), and various cellulose binding domains (U.S. Patent Nos. 5,496,934; 5,202,247; 5,137,819; Tomme *et al.*, 1994, Protein Eng. 7:117-123), etc. Other affinity labels may impart fluorescent properties to an α2M polypeptide, *e.g.*, portions of green fluorescent protein and the like. Other possible affinity labels are short amino acid sequences to which monoclonal antibodies are available, such as but not limited to the following well known examples, the FLAG epitope, the myc epitope at amino acids 408-439, the influenza virus hemagglutinin (HA) epitope. Other affinity labels are recognized by specific binding partners and thus facilitate isolation by affinity binding to the binding partner which can be immobilized onto a solid support. Some affinity labels may afford the α2M polypeptide novel structural properties, such as the ability to form multimers. Dimerization of an α2M polypeptide with a bound peptide may increase avidity of interaction between the α2M polypeptide and its partner in the course of antigen presentation. These affinity labels are usually derived from proteins that normally exist as homopolymers. Affinity labels such as the extracellular domains of CD8 (Shiue *et al.*, 1988, J. Exp. Med. 168:1993-2005), or CD28 (Lee *et al.*, 1990, J. Immunol. 145:344-352), or portions of the immunoglobulin molecule containing sites for interchain disulfide bonds, could lead to the formation of multimers. As will be appreciated by those skilled in the art, many methods can be used to obtain the coding region of the above-mentioned affinity labels, including but not limited to, DNA cloning, DNA amplification, and synthetic methods. Some of the affinity labels and reagents for their detection and isolation are available commercially.

A preferred affinity label is a non-variable portion of the immunoglobulin molecule. Typically, such portions comprise at least a functionally operative CH2 and CH3 domain of the constant region of an immunoglobulin heavy chain. Fusions are also made using the carboxyl terminus of the Fc portion of a constant domain, or a region immediately amino-

terminal to the CH1 of the heavy or light chain. Suitable immunoglobulin-based affinity label may be obtained from IgG-1, -2, -3, or -4 subtypes, IgA, IgE, IgD, or IgM, but preferably IgG1. Preferably, a human immunoglobulin is used when the α2M polypeptide is intended for *in vivo* use for humans. Many DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries. See, for example, Adams *et al.*, Biochemistry, 1980, 19:2711-2719; Gough *et al.*, 1980, Biochemistry, 19:2702-2710; Dolby *et al.*, 1980, Proc. Natl. Acad. Sci. U.S.A., 77:6027-6031; Rice *et al.*, 1982, Proc. Natl. Acad. Sci. U.S.A., 79:7862-7865; Falkner *et al.*, 1982, Nature, 298:286-288; and Morrison *et al.*, 1984, Ann. Rev. Immunol, 2:239-256. Because many immunological reagents and labeling systems are available for the detection of immunoglobulins, the α2M polypeptide–Ig fusion protein can readily be detected and quantified by a variety of immunological techniques known in the art, such as the use of enzyme-linked immunosorbent assay (ELISA), immunoprecipitation, fluorescence activated cell sorting (FACS), etc. Similarly, if the affinity label is an epitope with readily available antibodies, such reagents can be used with the techniques mentioned above to detect, quantitate, and isolate the α2M polypeptide containing the affinity label. In many instances, there is no need to develop specific antibodies to the α2M polypeptide.

A particularly preferred embodiment is a fusion of an α2M polypeptide to the hinge, the CH2 and CH3 domains of human immunoglobulin G-1 (IgG-1; see Bowen *et al.*,1996, J. Immunol. 156:442-49). This hinge region contains three cysteine residues which are normally involved in disulfide bonding with other cysteines in the Ig molecule. Since none of the cysteines are required for the peptide to function as a tag, one or more of these cysteine residues may optionally be substituted by another amino acid residue, such as for example, serine.

Various leader sequences known in the art can be used for the efficient secretion of α2M polypeptide from bacterial and mammalian cells (von Heijne, 1985, J. Mol. Biol. 184:99-105). Leader peptides are selected based on the intended host cell, and may include bacterial, yeast, viral, animal, and mammalian sequences. For example, the herpes virus glycoprotein D leader peptide is suitable for use in a variety of mammalian cells. A preferred leader peptide for use in mammalian cells can be obtained from the V-J2-C region of the mouse immunoglobulin kappa chain (Bernard *et al.*, 1981, Proc. Natl. Acad. Sci. 78:5812-5816). Preferred leader sequences for targeting α2M polypeptide expression in bacterial cells include, but are not limited to, the leader sequences of the *E.coli* proteins OmpA (Hobom *et al.*, 1995, Dev. Biol. Stand. 84:255-262), Pho A (Oka *et al.*, 1985, Proc. Natl. Acad. Sci 82:7212-16), OmpT (Johnson *et al.*, 1996, Protein Expression 7:104-113), LamB and OmpF (Hoffman & Wright, 1985, Proc. Natl. Acad. Sci. USA 82:5107-5111), β-lactamase (Kadonaga *et al.*, 1984, J. Biol. Chem. 259:2149-54), enterotoxins (Morioka-Fujimoto *et al.*,

1991, J. Biol. Chem. 266:1728-32), and the *Staphylococcus aureus* protein A (Abrahmsen *et al.*, 1986, Nucleic Acids Res. 14:7487-7500), and the *B. subtilis* endoglucanase (Lo *et al.*, Appl. Environ. Microbiol. 54:2287-2292), as well as artificial and synthetic signal sequences (MacIntyre *et al.*, 1990, Mol. Gen. Genet. 221:466-74; Kaiser *et al.*, 1987, Science, 235:312-317).

DNA sequences encoding a desired affinity label or leader peptide, which may be readily obtained from libraries, produced synthetically, or may be available from commercial suppliers, are suitable for the practice of this invention. Such methods are well known in the art.

5.1.1.2 RECOMBINANT EXPRESSION

In various embodiments of the invention, sequences encoding an α2M polypeptide are inserted into an expression vector for propagation and expression in recombinant cells.

An expression construct, as used herein, refers to a nucleotide sequence encoding an α2M polypeptide operably associated with one or more regulatory regions which allows expression of the α2M polypeptide in an appropriate host cell. "Operably-associated" refers to an association in which the regulatory regions and the α2M polypeptide sequence to be expressed are joined and positioned in such a way as to permit transcription, and ultimately, translation.

Vectors based on *E. coli* are the most popular and versatile systems for high level expression of foreign proteins (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* may include but not limited to *lac*, *trp*, *lpp*, *phoA*, *recA*, *tac*, $\lambda P_{L,}$ and phage T3 and T7 promoters (Makrides, 1996, Microbiol Rev, 60:512-538). Non-limiting examples of prokaryotic expression vectors may include the λgt vector series such as λgt11 (Huynh *et al.*, 1984 in "DNA Cloning Techniques", Vol. I: A Practical Approach (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier *et al.*, 1990, Methods Enzymol., 185:60-89). However, a potential drawback of a prokaryotic host-vector system is the inability to perform many of the post-translational processing events of mammalian cells. Thus, an eukaryotic host-vector system is preferred, a mammalian host-vector system is more preferred, and a human host-vector system is the most preferred.

The regulatory regions necessary for transcription of the α2M polypeptide can be provided by the expression vector. A translation initiation codon (ATG) may also be provided to express a nucleotide sequence encoding an α2M polypeptide that lacks an initiation codon. In a compatible host-construct system, cellular proteins required for transcription, such as RNA polymerase and transcription factors, will bind to the regulatory regions on the expression construct to effect transcription of the α2M polypeptide sequence

in the host organism. The precise nature of the regulatory regions needed for gene expression may vary from host cell to host cell. Generally, a promoter is required which is capable of binding RNA polymerase to initiate the transcription of an operably-associated nucleic acid sequence. Such regulatory regions may include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, the cap site, a CAAT box, and the like. The non-coding region 3' to the coding sequence may contain transcriptional termination regulatory sequences, such as terminators and polyadenylation sites.

Both constitutive and inducible regulatory regions may be used for expression of the α2M polypeptide. It may be desirable to use inducible promoters when the conditions optimal for growth of the recombinant cells and the conditions for high level expression of the α2M polypeptide are different. Examples of useful regulatory regions are provided in the next section below.

For expression of α2M polypeptides in mammalian host cells, a variety of regulatory regions can be used, for example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter. Inducible promoters that may be useful in mammalian cells include but are not limited to those associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the β-interferon gene, and the α2M70 gene (Williams *et al.*, 1989, Cancer Res. 49:2735-42 ; Taylor *et al.*, 1990, Mol. Cell Biol., 10:165-75).

The following animal regulatory regions, which exhibit tissue specificity and have been utilized in transgenic animals, can also be used in tumor cells of a particular tissue type: elastase I gene control region which is active in pancreatic acinar cells (Swift *et al.*, 1984, Cell 38:639-646; Ornitz *et al.*, 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl *et al.*, 1984, Cell 38:647-658; Adames *et al.*, 1985, Nature 318:533-538; Alexander *et al.*, 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder *et al.*, 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert *et al.*, 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf *et al.*, 1985, Mol. Cell. Biol. 5:1639-1648; Hammer *et al.*, 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey *et al.*, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, 1985, Nature 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94; myelin basic protein gene control region which is active

in oligodendrocyte cells in the brain (Readhead *et al.*, 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason *et al.*, 1986, Science 234:1372-1378).

The efficiency of expression of the α2M polypeptide in a host cell may be enhanced by the inclusion of appropriate transcription enhancer elements in the expression vector, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, β-actin (see Bittner *et al.*, 1987, Methods in Enzymol. 153:516-544; Gorman, 1990, Curr. Op. in Biotechnol. 1:36-47).

The expression vector may also contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences may include but are not limited to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA. It may also be advantageous to use shuttle vectors that can be replicated and maintained in at least two types of host cells.

In addition, the expression vector may contain selectable or screenable marker genes for initially isolating or identifying host cells that contain DNA encoding an α2M polypeptide. For long term, high yield production of α2M polypeptide–antigenic molecule complexes, stable expression in mammalian cells is preferred. A number of selection systems may be used for mammalian cells, including, but not limited, to the Herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:817) genes can be employed in *tk*$^-$, *hgprt*$^-$ or *aprt*$^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (*dhfr*), which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:3567; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Other selectable markers, such as but not limited to histidinol and Zeocin™ can also be used.

In order to insert the α2M polypeptide DNA sequence into the cloning site of a vector, DNA sequences with regulatory functions, such as promoters, must be attached to DNA sequences encoding the α2M peptide-binding region. To do this, linkers or adapters providing the appropriate compatible restriction sites may be ligated to the ends of cDNA or synthetic DNA encoding an α2M polypeptide, by techniques well known in the art (Wu *et*

al., 1987, Methods in Enzymol 152:343-349). Cleavage with a restriction enzyme can be followed by modification to create blunt ends by digesting back or filling in single-stranded DNA termini before ligation. Alternatively, a desired restriction enzyme site can be introduced into a fragment of DNA by amplification of the DNA by use of PCR with primers containing the desired restriction enzyme site.

An expression construct comprising an α2M polypeptide sequence operably associated with regulatory regions can be directly introduced into appropriate host cells for expression and production of α2M polypeptide-antigenic molecule complexes without further cloning (see, for example, U.S. Patent No. 5,580,859). The expression constructs may also contain DNA sequences that facilitate integration of the α2M polypeptide sequence into the genome of the host cell, *e.g.*, via homologous recombination. In this instance, it is not necessary to employ an expression vector comprising a replication origin suitable for appropriate host cells in order to propagate and express the α2M polypeptide in the host cells.

Expression constructs containing cloned nucleotide sequence encoding α2M polypeptides can be introduced into the host cell by a variety of techniques known in the art, including but not limited to, for prokaryotic cells, bacterial transformation (Hanahan, 1985, in DNA Cloning, A Practical Approach, 1:109-136), and for eukaryotic cells, calcium phosphate mediated transfection (Wigler *et al.*, 1977, Cell 11:223-232), liposome-mediated transfection (Schaefer-Ridder *et al.*, 1982, Science 215:166-168), electroporation (Wolff *et al.*, 1987, Proc Natl Acad Sci 84:3344), and microinjection (Cappechi, 1980, Cell 22:479-488). Co-expression of an α2M polypeptide and an antigenic molecule in the same host cell can be achieved by essentially the same methods.

For long term, high yield production of properly processed α2M polypeptides or α2M polypeptide–antigenic molecule complexes, stable expression in mammalian cells is preferred. Cell lines that stably express α2M polypeptides or α2M polypeptide–antigenic molecule complexes may be engineered by using a vector that contains a selectable marker. By way of example but not limitation, following the introduction of the expression constructs, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the expression construct confers resistance to the selection and optimally allows cells to stably integrate the expression construct into their chromosomes and to grow in culture and to be expanded into cell lines. Such cells can be cultured for a long period of time while α2M polypeptide is expressed continuously.

The recombinant cells may be cultured under standard conditions of temperature, incubation time, optical density, and media composition. Alternatively, a recombinant antigenic cells may be cultured under conditions emulating the nutritional and physiological

requirements of the cancer cell or infected cell. However, conditions for growth of recombinant cells may be different from those for expression of α2M polypeptides and antigenic proteins. Modified culture conditions and media may also be used to enhance production of α2M–antigenic molecule complexes. Any techniques known in the art may be applied to establish the optimal conditions for producing α2M polypeptide or α2M polypeptide–antigenic molecule complexes.

5.1.1.3 PURIFICATION METHODS FOR RECOMBINANT α2M POLYPEPTIDES

Generally, the α2M polypeptides of the invention can be recovered and purified from recombinant cell cultures by known methods, including ammonium sulfate precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, immunoaffinity chromatography, hydroxyapatite chromatography, and lectin chromatography.

The invention provides methods for purification of recombinant α2M polypeptides by affinity purification, based on the properties of the affinity label present on the α2M polypeptide. One approach is based on specific molecular interactions between a tag and its binding partner. The other approach relies on the immunospecific binding of an antibody to an epitope present on the tag. The principle of affinity chromatography well known in the art is generally applicable to both of these approaches.

Described below are several methods based on specific molecular interactions of a tag and its binding partner.

A method that is generally applicable to purifying recombinant α2Ms that are fused to the constant regions of immunoglobulin is protein A affinity chromatography, a technique that is well known in the art. Staphylococcus protein A is a 42 kD polypeptide that binds specifically to a region located between the second and third constant regions of heavy chain immunoglobulins. Because of the Fc domains of different classes, subclasses and species of immunoglobulins, affinity of protein A for human Fc regions is strong, but may vary with other species. Subclasses that are less preferred include human IgG-3, and most rat subclasses. For certain subclasses, protein G (of Streptococci) may be used in place of protein A in the purification. Protein-A sepharose (Pharmacia or Biorad) is a commonly used solid phase for affinity purification of antibodies, and can be used essentially in the same manner for the purification of α2M polypeptide fused to an immunoglobulin Fc fragment. Secreted α2M polypeptide present in cell supernatant binds specifically to protein A on the solid phase, while the contaminants are washed away. Bound α2M polypeptide can be eluted by various buffer systems known in the art, including a succession of citrate, acetate and glycine-HCl buffers which gradually lowers the pH. This method is less

preferred if the recombinant cells also produce antibodies which will be copurified with the α2M polypeptide. See, for example, Langone, 1982, J. Immunol. meth. 51:3; Wilchek *et al.*, 1982, Biochem. Intl. 4:629; Sjobring *et al.*, 1991, J. Biol. Chem. 26:399; page 617-618, in Antibodies A Laboratory Manual, edited by Harlow and Lane, Cold Spring Harbor laboratory, 1988.

Alternatively, a polyhistidine tag may be used, in which case, the α2M polypeptide can be purified by metal chelate chromatography. The polyhistidine tag, usually a sequence of six histidines, has a high affinity for divalent metal ions, such as nickel ions (Ni^{2+}), which can be immobilized on a solid phase, such as nitrilotriacetic acid matrices. Polyhistidine has a well characterized affinity for Ni^{2+}-NTA-agarose, and can be eluted with either of two mild treatments: imidazole (0.1-0.2 M) will effectively compete with the resin for binding sites; or lowering the pH just below 6.0 will protonate the histidine side-chains and disrupt the binding. The purification method comprises loading the cell culture supernatant onto the Ni^{2+}-NTA-agarose column, washing the contaminants through, and eluting the α2M polypeptide with imidazole or weak acid. Ni^{2+}-NTA-agarose can be obtained from commercial suppliers such as Sigma (St. Louis) and Qiagen. Antibodies that recognize the polyhistidine tag are also available which can be used to detect and quantify the α2M polypeptide.

Another exemplary affinity label that can be used is the glutathione-S-transferase (GST) sequence, originally cloned from the helminth, *Schistosoma japonicum*. In general, an α2M–GST fusion expressed in a prokaryotic host cell, such as *E. coli*, can be purified from the cell culture supernatant by absorption with glutathione agarose beads, followed by elution in the presence of free reduced glutathione at neutral pH. Denaturing conditions are not required at any stage during purification, and therefore, it may be desirable for use in the loading of immobilized α2M polypeptides with antigenic peptides. Moreover, since GST is known to form dimers under certain conditions, dimeric α2M polypeptides may be obtained. See, Smith, 1993, Methods Mol. Cell Bio. 4:220-229.

Another useful affinity label that can be used is the maltose binding protein (MBP) of *E. coli*, which is encoded by the *mal*E gene. The secreted α2M polypeptide-MBP present in the cell supernatant binds to amylose resin while contaminants are washed away. The bound α2M polypeptide-MBP is eluted from the amylose resin by maltose. See, for example, Guan *et al.*, 1987, Gene 67:21-30.

The second approach for purifying α2M polypeptide is applicable to affinity labels that contain an epitope for which polyclonal or monoclonal antibodies are available. Various methods known in the art for purification of protein by immunospecific binding, such as immunoaffinity chromatography, and immunoprecipitation, can be used. See, for example, Chapter 13 in "Antibodies A Laboratory Manual", 1988, Harlow and Lane, (eds.), Cold

Spring Harbor Laboratory, N.Y. and Chapter 8, Sections I and II, in "Current Protocols in Immunology", 1991, Coligan *et al.* (eds.), John Wiley, the disclosure of which are both incorporated by reference herein.

The embodiments described above may be used to recover and purify α2M polypeptide–antigenic molecule complexes from the cell culture medium of mammalian cells, such as human cells expressing an α2M polypeptide of the invention. The methods can be adapted to perform medium and large scale purification of an α2M polypeptide and/or α2M-antigenic molecule complexes. Methods that do not require lowering pH or denaturing conditions are most preferred for purification of α2M polypeptide-antigenic molecule complexes. The methods may be used to isolate α2M polypeptides from eukaryotic cells, for example, cancer cells, tissues, isolated cells, or immortalized eukaryote cell lines infected with an intracellular pathogen, or cells obtained from a subject infected with a pathogen.

5.1.1.4 HOST–VECTOR SYSTEMS

Described herein are systems of vectors and host cells that can be used for the expression of α2M polypeptides. A variety of expression vectors may be used in the present invention which include, but are not limited to, plasmids, cosmids, phage, phagemids, or modified viruses. Typically, such expression vectors comprise a functional origin of replication for propagation of the vector in an appropriate host cell, one or more restriction endonuclease sites for insertion of the α2M polypeptide gene sequence, and one or more selection markers. The expression vector must be used with a compatible host cell which may be derived from a prokaryotic or an eukaryotic organism including but not limited to bacteria, yeasts, insects, mammals, and humans.

Expression constructs and vectors are introduced into host cells for the purpose of producing an α2M polypeptide. Any cell type that can produce α2Ms and is compatible with the expression vector may be used, including those that have been cultured *in vitro* or genetically engineered. Host cells may be obtained from normal or affected subjects, including healthy humans, cancer patients, and patients with an infectious disease, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. A host cell may be chosen which modifies and processes the expressed gene products in a specific fashion similar to the way the recipient processes α2Ms. For the purpose of producing large amounts of α2M, it is preferable that the type of host cell used in the present invention has been used for expression of heterologous genes, and is reasonably well characterized and developed for large-scale

production processes. In a specific embodiment, the host cells are from the same patient to whom α2M polypeptide–antigenic molecule complexes or recombinant cells expressing α2M polypeptide–antigenic molecule complexes are going to be administered. Otherwise said, the cells used to express the α2M polypeptide and used subsequently to administer immunotherapy to a subject are autologous to the subject.

Preferred mammalian host cells include but are not limited to those derived from humans, monkeys and rodents, (see, for example, Kriegler M. in "Gene Transfer and Expression: A Laboratory Manual", New York, Freeman & Co. 1990), such as monkey kidney cell line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293, 293-EBNA, or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol., 36:59, 1977; baby hamster kidney cells (BHK, ATCC CCL 10); chinese hamster ovary-cells-DHFR (CHO, Urlaub and Chasin. Proc. Natl. Acad. Sci. 77; 4216, 1980); mouse sertoli cells (Mather, Biol. Reprod. 23:243-251, 1980); mouse fibroblast cells (NIH-3T3), monkey kidney cells (CVI ATCC CCL 70); african green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor cells (MMT 060562, ATCC CCL51). Exemplary cancer cell types used for demonstrating the utility of recombinant cells (producing α2M polypeptide-antigenic molecule complexes) as a cancer vaccine are provided as follows: mouse fibroblast cell line, NIH3T3, mouse Lewis lung carcinoma cell line, LLC, mouse mastocytoma cell line, P815, mouse lymphoma cell line, EL4 and its ovalbumin transfectant, E.G7, mouse melanoma cell line, B16F10, mouse fibrosarcoma cell line, MC57, and human small cell lung carcinoma cell lines, SCLC#2 and SCLC#7.

A number of viral-based expression systems may also be utilized with mammalian cells to produce α2M polypeptides. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer *et al.*, 1979, Cell 17:725), adenovirus (Van Doren *et al.*, 1984, Mol Cell Biol 4:1653), adeno-associated virus (McLaughlin *et al.*, 1988, J Virol 62:1963), and bovine papillomas virus (Zinn *et al.*, 1982, Proc Natl Acad Sci 79:4897). In cases where an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control region, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts (see *e.g.*, Logan and Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81:3655-3659).

Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been developed for recombinant gene expression which exist as stable, multicopy (20-300 copies/cell) extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene construct is transfected into cultured mammalian cells, for example, by the techniques of calcium phosphate coprecipitation or electroporation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant selectable marker, such as histidinol and G418 resistance. For example, BPV vectors such as pBCMGSNeo and pBCMGHis may be used to express α2M polypeptide sequences (Karasuyama *et al.*, Eur. J. Immunol. 18:97-104; Ohe *et al.*, Human Gene Therapy, 6:325-33) which may then be transfected into a diverse range of cell types for expression of the α2M polypeptide.

Alternatively, the vaccinia 7.5K promoter may be used (see, *e.g.*, Mackett *et al.*, 1982, Proc. Natl. Acad. Sci. (USA) 79:7415-7419; Mackett *et al.*, 1984, J. Virol. 49:857-864; Panicali *et al.*, 1982, Proc. Natl. Acad. Sci. 79:4927-4931.) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) may be used. Such vectors can be used with a broad range of human host cells, *e.g.*, EBO-pCD (Spickofsky *et al.*, 1990, DNA Prot Eng Tech 2:14-18), pDR2 and λDR2 (available from Clontech Laboratories).

α2M polypeptides may also be made with a retrovirus-based expression system. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. In retroviruses such as Moloney murine leukemia virus, most of the viral gene sequences can be removed and replaced with nucleic acid sequences encoding α2M, while the missing viral functions can be supplied in *trans*. The host range for infection by a retroviral vector can also be manipulated by the choice of envelope used for vector packaging.

For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The α2M polypeptide DNA is inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR comprises a promoter, including but not limited to an LTR promoter, an R region, a U5 region and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers may also be included in the expression vector to facilitate selection of infected cells (see

McLauchlin *et al.*, 1990, Prog. Nucleic Acid Res. and Molec. Biol. 38:91-135; Morgenstern *et al.*, 1990, Nucleic Acid Res. 18:3587-3596; Choulika *et al.*, 1996, J. Virol 70:1792-1798; Boesen *et al.*, 1994, Biotherapy 6:291-302; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114).

Other useful eukaryotic host-vector system may include yeast and insect systems. In yeast, a number of vectors containing constitutive or inducible promoters may be used with *Saccharomyces cerevisiae* (baker's yeast), *Schizosaccharomyces pombe* (fission yeast), *Pichia pastoris,* and *Hansenula polymorpha* (methylotropic yeasts). For a review see, "Current Protocols in Molecular Biology", Vol. 2, 1988, Ausubel *et al.* (eds.), Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant *et al.*, 1987, Expression and Secretion Vectors for Yeast, 1987, in "Methods in Enzymology", Wu and Grossman (eds.), Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, in "Methods in Enzymology", Berger and Kimmel (eds.), Acad. Press, N.Y., Vol. 152, pp. 673-684; and "The Molecular Biology of the Yeast *Saccharomyces*", 1982, Strathern *et al.* (eds.), Cold Spring Harbor Press, Vols. I and II.

In an insect system a baculovirus, *Autographa californica* nuclear polyhidrosis virus (AcNPV), can be used as a vector to express an α2M polypeptide in *Spodoptera frugiperda* cells. The α2M polypeptide DNA may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). These recombinant viruses are then used to infect host cells in which the inserted DNA is expressed (see, *e.g.*, Smith *et al.*, 1983, J. Virol. 46:584; Smith, U.S. Patent No. 4,215,051).

Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences by techniques well known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel *et al.*, Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

5.1.1.5 SYNTHETIC PRODUCTION

An alternative to producing α2M by recombinant techniques is peptide synthesis. For example, a peptide corresponding to a portion of an α2M comprising the substrate-binding domain, or which binds peptides *in vitro*, can be synthesized by use of a peptide synthesizer. Conventional peptide synthesis may be used or other synthetic protocols well known in the art.

In addition, analogs and derivatives of α2M polypeptides can be chemically synthesized. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the α2M sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogs in general.

Peptides having α2M amino acid sequences, or a fragment, analog, mutant or derivative thereof, may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxyl group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

Purification of the resulting α2M polypeptides accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5.2 ANTIGENIC COMPLEXES COMPRISING α2M POLYPEPTIDES

5.2.1 ISOLATION OF INTRACELLULAR COMPLEXES OF α2M POLYPEPTIDES WITH ANTIGENIC MOLECULES

Described herein are methods for purifying α2M polypeptides or α2M polypeptide–antigenic molecule complexes of the invention from recombinant cells, and, with minor modifications known in the art, the α2M polypeptide or α2M–antigenic molecule complexes from the cell culture. Recombinant cells include, for example, cells expressing antigenic molecules and recombinantly expressing an α2M polypeptide. Such cells may be derived from a variety of sources, including, but not limited to, cells infected with an infectious agent and cancer cells.

The invention provides methods for purification of recombinant α2M polypeptide–antigenic molecule complexes by affinity purification, based on the properties of the affinity label present on the α2M polypeptide. One approach is based on specific molecular interactions between a tag and its binding partner. The other approach relies on the immunospecific binding of an antibody to an epitope present on the tag. The principle of affinity chromatography well known in the art is generally applicable to both of these approaches.

To produce α2M polypeptide–antigenic molecule complexes, a nucleotide sequence encoding an α2M polypeptide can be introduced into a cell. When an antigenic molecule is present in the cell, the α2M polypeptide can associate intracellularly with the antigenic molecule, forming a covalent or a noncovalent complex of α2M polypeptide and the antigenic molecule. Cells into which an α2M polypeptide-encoding nucleotide sequence can be introduced, include, but are not limited to, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc. The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art. In a specific embodiment, an expression construct comprising a nucleic acid sequence encoding the α2M polypeptide is introduced into an antigenic cell. As used herein, antigenic cells may include cells that are infected with an infectious agent or pathogen, cells infected with non-infectious or non-pathogenic forms of an infectious agent or pathogen (*e.g.*, by use of a helper infectious agent), cells infected by or engineered to express an attenuated form of an infectious agent or a non-pathogenic or replication-deficient variant of a pathogen, pre-neoplastic cells that are infected with a cancer-causing infectious agent, such as a virus, but which are not yet neoplastic; or

antigenic cells that have been exposed to a mutagen or cancer-causing agent, such as, for example DNA-damaging agents, radiation, etc. Other cells that can be used are pre-neoplastic cells which are in transition from a normal to a neoplastic form as characterized by morphology, physiological or biochemical functions. Preferably, the cancer cells and pre-neoplastic cells used in the methods of the invention are of mammalian origin. Mammals contemplated by this aspect of the invention include humans, companion animals (*e.g.*, dogs and cats), livestock animals (*e.g.*, sheep, cattle, goats, pigs and horses), laboratory animals (*e.g.*, mice, rats and rabbits), and captive or free wild animals.

In various embodiments, any cancer cell, preferably a human cancer cell, can be used in the present methods for producing α2M polypeptide–antigenic molecule complexes. The cancer cells provide the antigenic peptides which become associated covalently or noncovalently with the expressed α2M polypeptide. α2M polypeptide-antigenic molecule complexes are then purified from the cells and used to treat such cancers. Cancers which can be treated or prevented with immunogenic compositions prepared by methods of the invention include, but are not limited to, tumors such as sarcomas and carcinomas. Examples of cancers that are amenable to the methods of the invention are listed in Section 5.6. Accordingly, any tissues or cells isolated from a pre-neoplastic lesion, a cancer, including cancer that has metastasized to multiple remote sites, can be used in the present method. For example, cells found in abnormally growing tissue, circulating leukemic cells, metastatic lesions as well as solid tumor tissue can be used.

In another embodiment, cell lines derived from a pre-neoplastic lesion, cancer tissues or cancer cells can also be used, provided that the cells of the cell line have at least one or more antigenic determinants in common with antigens on the target cancer cells. Cancer tissues, cancer cells, cells infected with a cancer-causing agent, other pre-neoplastic cells, and cell lines of human origin are preferred.

Cancer and pre-neoplastic cells can be identified by any method known in the art. For example, cancer cells can be identified by morphology, enzyme assays, proliferation assays, cytogenetic characterization, DNA mapping, DNA sequencing, the presence of cancer-causing virus, or a history of exposure to mutagen or cancer-causing agent, imaging, etc. Cancer cells may also be obtained by surgery, endoscopy, or other biopsy techniques. If some distinctive characteristics of the cancer cells are known, they can also be obtained or purified by any biochemical or immunological methods known in the art, such as but not limited to affinity chromatography, and fluorescence activated cell sorting (*e.g.*, with fluorescently tagged antibody against an antigen expressed by the cancer cells).

Cancer tissues, cancer cells or cell lines may be obtained from a single individual or pooled from several individuals. It is not essential that clonal, homogeneous, or purified population of cancer cells be used. It is also not necessary to use cells of the ultimate target

in vivo (*e.g.*, cells from the tumor of the intended recipient), so long as at least one or more antigenic determinants on the target cancer cells is present on the cells used for expression of the α2M polypeptide. In addition, cells derived from distant metastases may be used to prepare an immunogenic composition against the primary cancer. A mixture of cells can be used provided that a substantial number of cells in the mixture are cancer cells and share at least one antigenic determinant with the target cancer cell. In a specific embodiment, the cancer cells to be used in expressing an α2M polypeptide are purified.

5.2.2 *IN VITRO* COMPLEXING

In another embodiment, complexes of α2M polypeptides and antigenic molecules are produced *in vitro*. Immunogenic α2M polypeptide – antigenic molecule complexes can be generated *in vitro* by any method known in the art for forming α2M polypeptide – antigenic molecule complexes. Procedures for forming such α2M–antigenic molecule complexes and methods for isolating antigenic peptides are described in detail herein.

Methods for formation *in vitro* of noncovalent immunogenic complexes are well known in the art. For example, such complexes can be generated *in vitro* by noncovalent complexing of an α2M polypeptide with an antigenic molecule using methods which have been previously described for noncovalent coupling of an HSP with an antigenic molecule (see *e.g.*, Blachere *et al.*, 1997, *supra*; PCT publication WO 97/10000, dated March 20, 1997). Preferably, the immunogenic molecular complex is not prepared by treatment with a protease, or with an activating agent such as ammonia or methyamine. In another preferred embodiment, the α2M molecule of the immunogenic molecular complex is not cleaved within the "bait" region. In yet another embodiment, the α2M polypeptide is not covalently associated with the antigenic molecule through a thioester linkage.

Methods for covalent coupling are also well known in the art (see, *e.g.*, Osada *et al.*, 1987, *supra*; Osada *et al.*, 1988, *supra*; Chu and Pizzo 1993, *supra*; Chu *et al.*, 1994, *supra*; Mitsuda *et al.*, 1993, *supra*). In one embodiment, for example, when an α2M polypeptide is mixed with protease,, During proteolytic activation of α2M, non-proteolytic ligands can become covalently bound to the activated thioesters. Non-proteolytic ligands can also be incorporated into the activated α2M molecule by ammonia or methylamine during reversal of the nucleophilic activation, employing heat (Grøn and Pizzo, 1998, Biochemistry, 37: 6009-6014). Such conditions that allow fortuitous trapping of peptides by α2M are employed to prepare the α2M polypeptide – antigenic molecule complexes of the invention.

For example, in various embodiments of the invention, an α2M polypeptide may be mixed with antigenic molecule in the presence of a protease, ammonia or other small amine nucleophiles such as methylamine and ethylamine. Non-limiting examples of proteases which may be used include trypsin, porcine pancreatic elastase (PEP), human neutrophil

elastase, cathepsin G, *S. aureus* V-8 proteinase trypsin, a-chymotrypsin, V8 protease, papain, and proteinase K (see Ausubel *et al.*, (eds.), in "Current Protocols in Molecular Biology", Greene Publishing Associates and Wiley Interscience, New York, 17.4.6-17.4.8).

In another embodiment for preparation of covalent α2M polypeptide–antigenic molecule complexes, α2M polypeptides and antigenic molecules are prepared, and then covalently coupled using, for example, chemical crosslinking. Chemical crosslinking methods are well known in the art. For example, in a preferred embodiment, glutaraldehyde crosslinking may be used. Glutaradehyde crosslinking has been used for formation of covalent complexes of peptides and hsps (see Barrios *et al.*, 1992, Eur. J. Immunol. 22: 1365-1372). In one embodiment, the following protocol is used. Optionally, α2M polypeptides may be pretreated with ATP or low pH prior to complexing, in order to remove any peptides that may be associated with the α2M polypeptide. Preferably, 1 mg of α2M polypeptide is crosslinked to 1 mg of peptide in the presence of 0.002% glutaraldehyde for 2 hours. Glutaraldehyde is removed by dialysis against phosphate buffered saline (PBS) overnight (Lussow *et al.*, 1991, Eur. J. Immunol. 21: 2297-2302).

Other methods for chemical crosslinking may also be used, in addition other methods for covalent attachment of proteins, such as photocrosslinking (see Current Protocols in Molecular Biology, Ausubel *et al.* (eds.), Greene Publishing Associates and Wiley Interscience, New York).

Antigenic molecules for covalent or noncovalent α2M polypeptide–antigenic molecule complexes may be isolated from various sources, chemically synthesized, or produced recombinantly. Such methods can be readily adapted for medium or large scale production of the immunotherapeutic or prophylactic vaccines of the invention.

Following complexing, the immunogenic α2M-antigenic molecule complexes can optionally be purified. In a preferred embodiment, such complexes are at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 100% nonconvalent complexes of α2M and the antigenic molecule. Such complexes may be assayed *in vitro* using, for example, the mixed lymphocyte target cell assay (MLTC) described below. Once immunogenic complexes have been isolated they can be optionally characterized further in animal models using the preferred administration protocols and excipients discussed below.

5.2.3. α2M – ANTIGENIC MOLECULE FUSION PROTEINS

In another embodiment, recombinant fusion proteins, comprised of α2M sequences linked to antigenic molecule sequences, may be used for immunotherapy. To produce such a recombinant fusion protein, an expression vector is constructed using nucleic acid sequences encoding α2M fused to sequences encoding an antigenic molecule, using recombinant methods known in the art, such as those described in Sections 5.1.1.1 and

5.1.1.2, above (see Suzue *et al.*, 1997, Proc. Natl. Acad. Sci. U.S.A. 94: 13146-51). α2M-antigenic peptide fusions are then expressed and isolated. By specifically designing the antigenic peptide portion of the molecule, such fusion proteins can be used to elicit an immune response and in immunotherapy against target cancer and infectious diseases.

5.2.4 SOURCES OF ANTIGENIC MOLECULES

Antigenic molecules, or antigenic portions thereof, specific to one or more types of cancer or infected cells, can be chosen from among those known in the art. Alternatively, such antigenic molecules can be selected for their antigenicity or their immunogenicity, as determined by immunoassays or by their ability to generate an immune response.

5.2.4.1 EXOGENOUS ANTIGENIC MOLECULES

Preferably, where it is desired to treat or prevent cancer, known tumor-specific antigenic molecules or fragments or derivatives thereof are used. For example, such tumor specific or tumor-associated antigenic molecules include but are not limited to KS 1/4 pan-carcinoma antigen (Perez and Walker, 1990, J. Immunol. 142:3662-3667; Bumal, 1988, Hybridoma 7(4):407-415); ovarian carcinoma antigen (CA125) (Yu, *et al.*, 1991, Cancer Res. 51(2):468-475); prostatic acid phosphate (Tailer, *et al.*, 1990, Nucl. Acids Res. 18(16):4928); prostate specific antigen (Henttu and Vihko, 1989, Biochem. Biophys. Res. Comm. 160(2):903-910; Israeli, *et al.*, 1993, Cancer Res. 53:227-230); melanoma-associated antigen p97 (Estin, *et al.*, 1989, J. Natl. Cancer Inst. 81(6):445-446); melanoma antigen gp75 (Vijayasardahl, *et al.*, 1990, J. Exp. Med. 171(4):1375-1380); high molecular weight melanoma antigen (Natali, *et al.*, 1987, Cancer 59:55-63) and prostate specific membrane antigen.

In a specific embodiment, an antigenic molecule or fragment or derivative thereof specific to a certain tumor is selected for complexing to α2M polypeptide and subsequent administration to a patient having that tumor.

In a preferred embodiment, where it is desired to treat or prevent viral diseases, molecules comprising epitopes of known viruses are used. For example, such antigenic epitopes may be prepared from viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), rinderpest, rhinovirus, echovirus, rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsackie virus, mumps virus, measles virus, rubella virus, polio virus, human immunodeficiency virus type I (HIV-I), and human immunodeficiency virus type II (HIV-II).

In another preferred embodiment, where it is desired to treat or prevent bacterial infections, molecules comprising epitopes of known bacteria are used. For example, such antigenic epitopes may be prepared from bacteria including, but not limited to, mycobacteria rickettsia, mycoplasma, neisseria and legionella.

In another preferred embodiment, where it is desired to treat or prevent protozoal infections, molecules comprising epitopes of known protozoa are used. For example, such antigenic epitopes may be prepared from protozoa including, but not limited to, leishmania, kokzidioa, and trypanosoma.

In yet another preferred embodiment, where it is desired to treat or prevent parasitic infections, molecules comprising epitopes of known parasites are used. For example, such antigenic epitopes may be from parasites including, but not limited to, chlamydia and rickettsia.

To determine immunogenicity or antigenicity of a putative antigen by detecting binding to antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, *in vivo* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, immunoprecipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one aspect, antibody binding is detected by detecting a label on the primary antibody. In another aspect, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further aspect, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are envisioned for use. In one embodiment for detecting immunogenicity, T cell-mediated responses can be assayed by standard methods, *e.g.*, *in vitro* cytoxicity assays or *in vivo* delayed-type hypersensitivity assays.

Potentially useful antigenic molecules, or derivatives thereof, can be identified by various criteria, such as the antigen's involvement in neutralization of a pathogen's infectivity (wherein it is desired to treat or prevent infection by such a pathogen) (Norrby, 1985, *Summary*, in Vaccines 85, Lerner, *et al.* (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, pp. 388-389), type or group specificity, recognition by patients' antisera or immune cells, and/or the demonstration of protective effects of antisera or immune cells specific for the antigen. In addition, where it is desired to treat or prevent a disease caused by pathogen, the antigen's encoded epitope should preferably display a small or no degree of antigenic variation in time or amongst different isolates of the same pathogen.

5.2.4.2 ANTIGENIC MOLECULES FROM α2M COMPLEXES

Antigenic peptides for complexing *in vitro* to α2M polypeptides of the invention can also be obtained from endogenous complexes of peptides and α2Ms. Two methods may be used to elute the peptide from an α2M-antigenic molecule complex. One approach involves incubating the α2M-antigenic molecule complex in the presence of ATP. The other approach involves incubating the complexes in a low pH buffer.

Briefly, the complex of interest is centrifuged through a Centricon 10 assembly (Millipore) to remove any low molecular weight material loosely associated with the complex. The large molecular weight fraction may be removed and analyzed by SDS-PAGE while the low molecular weight may be analyzed by HPLC as described below. In the ATP incubation protocol, the α2M-antigenic molecule complex in the large molecular weight fraction is incubated with 10mM ATP for 30 minutes at room temperature. In the low pH protocol, acetic acid or trifluoroacetic acid (TFA) is added to the α2M-antigenic molecule complex to give a final concentration of 10% (vol/vol) and the mixture incubated at room temperature or in a boiling water bath or any temperature in between, for 10 minutes.

The resulting samples are centrifuged through a Centricon10 assembly as mentioned previously. The high and low molecular weight fractions are recovered. The remaining large molecular weight α2M-antigenic molecule complexes can be reincubated with ATP or low pH to remove any remaining peptides.

The resulting lower molecular weight fractions are pooled, concentrated by evaporation and dissolved in 0.1% TFA. The dissolved material is then fractionated by reverse phase high pressure liquid chromatography (HPLC) using for example a VYDAC C18 reverse phase column equilibrated with 0.1% TFA. The bound material is then eluted at a flow rate of about 0.8 ml/min by developing the column with a linear gradient of 0 to 80% acetonitrile in 0.1% TFA. The elution of the peptides can be monitored by OD_{210} and the fractions containing the peptides collected.

5.2.4.3 PEPTIDE ANTIGENS FROM MHC COMPLEXES

Peptides bound to MHC molecules *in vivo* can also be used *in vitro* to form complexes with α2M polypeptides of the invention. The isolation of potentially immunogenic peptides from MHC molecules is well known in the art and so is not described in detail herein (*see*, Falk, *et al.*, 1990, Nature 348:248-251; Rotzsche, at al., 1990, Nature 348:252-254; Elliott, *et al.*, 1990, Nature 348:191-197; Falk, *et al.*, 1991, Nature 351:290-296; Demotz, *et al.*, 1989, Nature 343:682-684; Rotzsche, *et al.*, 1990, Science 249:283-287), the disclosures of which are incorporated herein by reference.

Briefly, MHC-antigenic molecule complexes may be isolated by a conventional immunoaffinity procedure. The peptides then may be eluted from the MHC-antigenic molecule complex by incubating the complexes in the presence of about 0.1% TFA in acetonitrile. The eluted peptides may be fractionated and purified by reverse phase HPLC, as before.

5.2.4.4 SYNTHETIC ANTIGENIC MOLECULES

The amino acid sequences of the peptides eluted from MHC molecules or α2M may be determined either by manual or automated amino acid sequencing techniques well known in the art. Once the amino acid sequence of a potentially protective peptide has been determined, the peptide may be synthesized in using conventional peptide synthesis or other protocols well known in the art.

Peptides having the same amino acid sequence as those isolated above may be synthesized by solid-phase peptide synthesis using procedures similar to those described by Merrifield, 1963, J. Am. Chem. Soc., 85:2149. During synthesis, N-α-protected amino acids having protected side chains are added stepwise to a growing polypeptide chain linked by its C-terminal and to an insoluble polymeric support i.e., polystyrene beads. The peptides are synthesized by linking an amino group of an N-α-deprotected amino acid to an α-carboxyl group of an N-α-protected amino acid that has been activated by reacting it with a reagent such as dicyclohexylcarbodiimide. The attachment of a free amino group to the activated carboxyl leads to peptide bond formation. The most commonly used N-α-protecting groups include Boc which is acid labile and Fmoc which is base labile. Details of appropriate chemistries, resins, protecting groups, protected amino acids and reagents are well known in the art and so are not discussed in detail herein (*See*, Atherton, *et al.*, 1989, Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, and Bodanszky, 1993, Peptide Chemistry, A Practical Textbook, 2nd Ed., Springer-Verlag).

Purification of the resulting peptides is accomplished using conventional procedures, such as preparative HPLC using gel permeation, partition and/or ion exchange chromatography. The choice of appropriate matrices and buffers are well known in the art and so are not described in detail herein.

5.2.4.5 RECOMBINANTLY PRODUCED ANTIGENIC MOLECULES

In a particular embodiment of the invention, a nucleotide sequence encoding a protein antigenic molecule or portions thereof can be introduced into a host cell for production of the antigenic molecule. The nucleotide sequence encoding any antigenic protein can be obtained and cloned into an expression vector for expression essentially by the same methods described for the cloning and expression of a nucleotide sequence encoding an α2M

polypeptide. The techniques are described in Sections 5.1.1.1 and 5.1.1.2, and are well known in the art. The recombinant antigenic protein or portions thereof can be purified by any methods appropriate for the protein, and then used to form complexes with α2M polypeptides *in vitro* as described in Section 5.2.2. Such an α2M polypeptide-antigenic molecule complex can be used as a vaccine to stimulate an immune response against the antigenic protein in a subject for the purpose of treatment or prevention of infectious diseases or cancer.

5.3 THERAPEUTIC APPLICATIONS FOR α2M COMPLEXES

The present invention encompasses the use of α2M polypeptides in methods for treatment of and prevention of infectious diseases and cancer. In various embodiments described in detail herein, an effective amount of a α2M polypeptide in a covalent or noncovalent complex with an antigenic molecule is administered to a patient for therapeutic purposes.

5.3.1 PREVENTION AND TREATMENT OF INFECTIOUS DISEASES

For treatment and prevention of infectious disease, α2M – antigenic molecule complexes are prepared from a cell that displays the antigenicity of an antigen of an infectious agent or pathogenic agent, and used as vaccines against the infectious disease. As will be appreciated by those skilled in the art, the protocols described herein may be used to isolate α2M polypeptide–antigenic molecule complexes from any cell that displays the antigenicity of an antigen of the infectious agent. For example, cells may be infected by the infectious agent itself, or alternatively, cells may be infected by or engineered to express an attenuated form of the infectious agent or a non-pathogenic or replication-deficient variant of the pathogen. In one embodiment, α2M– antigenic molecule complexes can be prepared from cells infected with non-infectious or non-pathogenic forms of the infectious agent (*e.g.*, by use of a helper infectious agent). In another embodiment, the α2M–antigenic molecule complexes of the invention may be prepared from cells infected with an intracellular pathogen. In another embodiment, α2M polypeptide-complexes can be prepared from cells that have been transformed by an intracellular pathogen. For example, immunogenic α2M polypeptide–antigenic molecule complexes may be isolated from eukaryotic cells transformed with a transforming virus such as SV40.

A preferred method for treatment or prevention of an infectious disease comprises introducing into a cell that displays the antigenicity of an infectious agent an expressible α2M polypeptide gene sequence, preferably as an expression gene construct. The α2M polypeptide gene sequence is manipulated by recombinant methods, such as those described

above in Sections 5.1.1.1 and 5.1.1.2 above, so that the α2M polypeptide gene sequence, in the form of an expression construct, located extrachromosomally or integrated in the chromosome, is suitable for expression of the α2M polypeptide in the recombinant cells. The recombinant cells containing the expression gene constructs are cultured under conditions such that α2M polypeptides encoded by the expression gene construct are expressed. Complexes of α2M polypeptides covalently or noncovalently associated with antigenic molecules of the infectious agent are purified from the cell culture or culture medium by the methods described in Section 5.2.1.

In various embodiments, α2M – antigenic molecule complexes are prepared from a cell genetically manipulated to express an α2M polypeptide, for example, tissues, isolated cells or immortalized eukaryotic cell lines infected with an intracellular pathogen. When immortalized animal cell lines are used as a source of the α2M polypeptide–antigenic molecule complex, it is important to use cell lines that can be infected with the pathogen of interest. In addition, it is preferable to use cells that are derived from the same species as the intended recipient of the vaccine. Techniques for introducing an expressible form of the α2M polypeptide gene sequences into these cell lines are described above in Section 5.1.1.2. If a pathogen is expected to cause lysis of the host cells, it is preferred to introduce the expressible α2M polypeptide gene sequence into the host cell prior to infecting the cells with the pathogen. For example, in order to prepare an α2M polypeptide–antigenic molecule complex for administration to humans that may be effective against HIV-1, the virus may be propagated in human cells which include, but are not limited to, human CD4+ T cells, HepG2 cells, and U937 promonocytic cells, which have already been transfected with an expressible α2M polypeptide sequence. Similarly, influenza viruses may be propagated in, for example, transfected human fibroblast cell lines and MDCK cells, and mycobacteria may be cultured in, for example, transfected human Schwaan cells. The cell supernatant containing α2M–antigenic molecule complex may be collected just prior to lysis of the host cell.

In a preferred aspect of the invention, the purified α2M – antigenic molecule complex vaccines may have particular utility in the treatment of human diseases caused by intracellular pathogens. It is appreciated, however, that the vaccines developed using the principles described herein will be useful in treating diseases of other mammals, for example, farm animals including: cattle; horses; sheep; goats; and pigs, and household pets including: cats; and dogs, that similarly are caused by intracellular pathogens.

In accordance with the methods described herein, vaccines may be prepared that stimulate an immune response, in particular a cytotoxic T cell responses, against cells infected with viruses including, but not limited to, hepatitis type A, hepatitis type B, hepatitis type C, influenza, varicella, adenovirus, HSV-I, HSV-II, rinderpest rhinovirus, echovirus,

rotavirus, respiratory syncytial virus, papilloma virus, papova virus, cytomegalovirus, echinovirus, arbovirus, huntavirus, coxsachie virus, mumps virus, measles virus, rubella virus, polio virus, HIV-I, and HIV-II. Similarly, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular bacteria, including, but not limited to, *Mycobacteria, Rickettsia, Mycoplasma, Neisseria* and *Legionella*. In addition, vaccines may also be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular protozoa, including, but not limited to, *Leishmani, Kokzidioa,* and *Trypanosoma*. Furthermore, vaccines may be prepared that stimulate cytotoxic T cell responses against cells infected with intracellular parasites including, but not limited to, *Chlamydia* and *Rickettsia*.

The effect of immunotherapy with modified α2M polypeptide–antigenic molecule complexes on progression of infectious diseases can be monitored by any methods known to one skilled in the art.

5.3.2 PREVENTION AND TREATMENT OF CANCER

There are many reasons why immunotherapy as provided by the covalent or noncovalent α2M polypeptide-antigenic molecule complexes or recombinant cells expressing α2M polypeptides prepared by the present invention is desired for use in cancer patients. First, if cancer patients are immunosuppressed, and surgery with anesthesia, and subsequent chemotherapy, may worsen the immunosuppression, then with appropriate immunotherapy in the preoperative period, this immunosuppression may be prevented or reversed. This could lead to fewer infectious complications and to accelerated wound healing. Second, tumor bulk is minimal following surgery and immunotherapy is most likely to be effective in this situation. A third reason is the possibility that tumor cells are shed into the circulation at surgery and effective immunotherapy applied at this time can eliminate these cells.

In a specific embodiment, the preventive and therapeutic utility of the invention is directed at enhancing the immunocompetence of the cancer patient either before surgery, at or after surgery, and at inducing tumor-specific immunity to cancer cells, with the objective being inhibition of cancer, and with the ultimate clinical objective being total cancer regression and eradication.

According to the invention, preferred methods of treatment or prevention of cancer comprise isolating cancer cells from one or more individual, preferably the individual in need of treatment, and introducing into such cells an expressible α2M polypeptide gene sequence, preferably as an expression gene construct. The α2M polypeptide gene sequence is manipulated by methods described above in Sections 5.1.1.1 and 5.1.1.2, such that the α2M polypeptide gene sequence, in the form of an expression construct, or intrachromosomally integrated, are suitable for expression of the α2M polypeptide in the recombinant cells. The

recombinant cells containing the expression gene constructs are cultured under conditions such that α2M polypeptides encoded by the expression gene construct are expressed by the recombinant host cells. Complexes of α2M polypeptides covalently or noncovalently associated with antigenic molecules of the cancer cell are purified from the cell culture or culture medium by the methods described in Section 5.2.1. Depending on the route of administration, the α2M polypeptide–antigenic molecule complexes are formulated accordingly as described in Section 5.7, and administered to the individual autologously (*e.g.*, to treat the primary cancer or metastases thereof), or to other individuals who are in need of treatment for cancer of a similar tissue type, or to individuals at enhanced risk of cancer due to familial history or environmental risk factors.

For example, treatment with α2M polypeptide – antigenic molecule complexes prepared as described above may be started any time after surgery. However, if the patient has received chemotherapy, α2M – antigenic molecule complexes are usually administered after an interval of four weeks or more so as to allow the immune system to recover. The therapeutic regimen may include weekly injections of the α2M polypeptide – antigenic molecule complex, dissolved in saline or other physiologically compatible solution. The route and site of injection is varied each time, for example, the first injection is given subcutaneously on the left arm, the second injection on the right arm, the third injection on the left abdominal region, the fourth injection on the right abdominal region, the fifth injection on the left thigh, the sixth injection on the right thigh, *etc.* The same site is repeated after a gap of one or more injections. In addition, injections are split and each half of the dose is administered at a different site on the same day. Overall, the first four to six injections are given at weekly intervals. Subsequently, two injections are given at two-week intervals, followed by a regimen of injections at monthly intervals.

Alternatively, recombinant tumor cells expressing α2M – antigenic molecule complexes can be used as a vaccine for injection into a patient to stimulate an immune response against the tumor cells or cells bearing tumor antigens. Autologous recombinant tumor cells stably expressing α2M polypeptide-antigenic molecule complexes are preferred. To determine the appropriate dose, the amount of α2M polypeptide-antigenic molecule complex produced by the recombinant cells is quantitated, and the number of recombinant cells used for vaccination is adjusted accordingly to assure a consistent level of expression *in vivo*. A preferred dose is the number of recombinant cells that can produce about 100 ng α2M polypeptide per 24 hours. For the safety of the patient, the recombinant tumor cells can be irradiated (12000 rad) immediately prior to injection into a patient. Irradiated cells do not proliferate, and can continue to express α2M polypeptide-antigenic molecule complexes for about 7-10 days which is sufficient to induce an immune response.

Cancers that can be treated or prevented by using covalent or noncovalent α2M–antigenic molecule complexes prepared by the methods of the present invention include, but not limited to human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

In a specific embodiment, the cancer is metastatic. In another specific embodiment, the patient having a cancer is immunosuppressed by reason of having undergone anti-cancer therapy (*e.g.*, chemotherapy radiation) prior to administration of the α2M – antigenic molecule complexes of the invention. In another specific embodiment, the cancer is a tumor.

The effect of immunotherapy with α2M polypeptide-antigenic molecule complexes on progression of neoplastic diseases can be monitored by any methods known to one skilled in the art, including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes in vitro; c) levels of tumor specific antigens, *e.g.*, carcinoembryonic (CEA) antigens; d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; e) changes in levels of putative biomarkers of risk for a particular cancer in individuals at high risk, and f) changes in the morphology of tumors using a sonogram. Other techniques that can also be used include scintigraphy and endoscopy.

The preventive effect of immunotherapy using α2M polypeptide-antigenic molecule complexes may also be estimated by determining levels of a putative biomarker for risk of a specific cancer. For example, in individuals at enhanced risk for prostate cancer, serum prostate-specific antigen (PSA) is measured by the procedure described by Brawer *et al.*,

1992, J. Urol. 147:841-845, and Catalona *et al.*, 1993, JAMA 270:948-958; or in individuals at risk for colorectal cancer, CEA is measured by methods known in the art; and in individuals at enhanced risk for breast cancer, 16-α-hydroxylation of estradiol is measured by the procedure described by Schneider *et al.*, 1982, Proc. Natl. Acad. Sci. USA 79:3047-3051. The references cited above are incorporated by reference herein in their entirety.

5.3.3 COMBINATION WITH ADOPTIVE IMMUNOTHERAPY

Adoptive immunotherapy refers to a therapeutic approach for treating infectious diseases or cancer in which immune cells are administered to a host with the aim that the cells mediate specific immunity, either directly or indirectly, to the infected cells or tumor cells and/or antigenic components, and result in treatment of the infectious disease or regression of the tumor, as the case may be (see U.S. Patent Application Serial No. 08/527,546, filed September 13, 1995, which is incorporated by reference herein in its entirety). α2M polypeptides may be used to sensitize antigen presenting cells (APCs) using in covalent or noncovalent complexes with antigenic (or immunogenic) molecules, for adoptive immunotherapy.

According to the invention, therapy by administration of α2M polypeptide–antigenic molecule complexes, using any desired route of administration, is combined with adoptive immunotherapy using APC sensitized with α2M polypeptide–antigenic molecule complexes. The α2M polypeptide–antigenic molecule complex-sensitized APC can be administered concurrently with α2M polypeptide–antigenic molecule complexes, or before or after administration of α2M polypeptide– antigenic molecule complexes. Furthermore, the mode of administration can be varied, including but not limited to, *e.g.*, subcutaneously, intravenously, intraperitoneally, intramuscularly, intradermally or mucosally.

5.3.3.1 SENSITIZATION OF ANTIGEN PRESENTING CELLS WITH α2M COMPLEXES

The antigen-presenting cells, including but not limited to macrophages, dendritic cells and B-cells, are preferably obtained by production *in vitro* from stem and progenitor cells from human peripheral blood or bone marrow as described by Inaba *et al.*, 1992, J. Exp. Med. 176:1693-1702. APC can be obtained by any of various methods known in the art. In a preferred aspect human macrophages are used, obtained from human blood cells.

By way of example, but not limitation, macrophages can be obtained as follows: Mononuclear cells are isolated from peripheral blood of a patient (preferably the patient to be treated), by Ficoll-Hypaque gradient centrifugation and are seeded on tissue culture dishes which are pre-coated with the patient's own serum or with other AB+ human serum. The

cells are incubated at 37°C for 1 hr, then non-adherent cells are removed by pipetting. To the adherent cells left in the dish, is added cold (4°C) 1 mM EDTA in phosphate-buffered saline and the dishes are left at room temperature for 15 minutes. The cells are harvested, washed with RPMI buffer and suspended in RPMI buffer. Increased numbers of macrophages may be obtained by incubating at 37°C with macrophage-colony stimulating factor (M-CSF); increased numbers of dendritic cells may be obtained by incubating with granulocyte-macrophage-colony stimulating factor (GM-CSF) as described in detail by Inaba, K., *et al.*, 1992, J. Exp. Med. 176:1693-1702.

APC are sensitized with α2M polypeptides covalently or noncovalently bound to antigenic molecules by incubating the cells *in vitro* with the complexes. The APC are sensitized with complexes of α2M polypeptide and antigenic molecules preferably by incubating *in vitro* with the α2M polypeptide–complex at 37°C for 15 minutes to 24 hours. By way of example but not limitation, $4x10^7$ macrophages can be incubated with 10 microgram α2M-antigenic molecule complexes per ml or 100 microgram α2M-antigenic molecule complexes per ml at 37°C for 15 mins to 24 hrs in 1 ml plain RPMI medium. The cells are washed three times and resuspended in a physiological medium preferably sterile, at a convenient concentration (*e.g.*, $1x10^7$/ml) for injection in a patient. In a preferred embodiment, the antigen presenting cells are autologous to the patient, that is, the patient into which the sensitized APCs are injected is the patient from which the APC were originally isolated,.

Optionally, the ability of sensitized APC to stimulate, for example, the antigen-specific, class I-restricted cytotoxic T-lymphocytes (CTL) can be monitored by their ability to stimulate CTLs to release tumor necrosis factor, and by their ability to act as targets of such CTLs.

5.3.3.2 REINFUSION OF SENSITIZED APC

The α2M polypeptide–antigen-sensitized APC are reinfused into the patient systemically, preferably intravenously, by conventional clinical procedures. These activated cells are reinfused, preferentially by systemic administration into the autologous patient. Patients generally receive from about 10^6 to about 10^{12} sensitized macrophages, depending on the condition of the patient. In some regimens, patients may optionally receive in addition a suitable dosage of a biological response modifier including but not limited to the cytokines IFN-α, IFN-γ, IL-2, IL-4, IL-6, TNF or other cytokine growth factor.

5.3.4 DETERMINATION OF IMMUNOGENICITY OF α2M-ANTIGEN MOLECULE COMPLEXES

In an optional procedure, the purified α2M polypeptide–antigenic molecule complexes can be assayed for immunogenicity using the mixed lymphocyte target culture assay (MLTC) well known in the art.

By way of example but not limitation, the following procedure can be used. Briefly, mice are injected subcutaneously with the candidate α2M polypeptide-antigenic molecule complexes. As a positive control another set of mice are immunized with whole cancer cells of the type from which the α2M polypeptides are derived. As a negative control, mice are injected with either α2M – antigenic molecule complexes isolated from normal, non-recombinant cells or whole cells (*i.e.*, antigenically distinct from the type of cell from which the α2M polypeptides are derived). The mice are injected twice, 7-10 days apart. The mice are injected twice, 7-10 days apart. Ten days after the last immunization, the spleens are removed and the lymphocytes released. The released lymphocytes may be restimulated subsequently in vitro by the addition of dead cells that expressed the complex of interest.

For example, $8x10^6$ immune spleen cells may be stimulated with $4x10^4$ mitomycin C treated or γ-irradiated (5-10,000 rads) pathogen-infected cells (or cells transfected with a gene encoding an antigen of the infectious agent, as the case may be), or tumor cells in 3ml RPMI medium containing 10% fetal calf serum. In certain cases 33% secondary mixed lymphocyte culture supernatant or interleukin 2 (IL-2) may be included in the culture medium as a source of T cell growth factors (See, Glasebrook *et al.*, 1980, J. Exp. Med. 151:876). To test the primary cytotoxic T cell response after immunization, spleen cells may be cultured without stimulation. In some experiments spleen cells of the immunized mice may also be restimulated with antigenically distinct cells, to determine the specificity of the cytotoxic T cell response.

Six days later the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay (See, Palladino *et al.*, 1987, Cancer Res. 47:5074-5079 and Blachere, at al., 1993, J. Immunotherapy 14:352-356). In this assay, the mixed lymphocyte culture is added to a target cell suspension to give different effector:target (E:T) ratios (usually 1:1 to 40:1). The target cells are prelabelled by incubating $1x10^6$ target cells in culture medium containing 200 mCi ^{51}Cr/ml for one hour at 37°C. The cells are washed three times following labeling. Each assay point (E:T ratio) is performed in triplicate and the appropriate controls incorporated to measure spontaneous ^{51}Cr release (no lymphocytes added to assay) and 100% release (cells lysed with detergent). After incubating the cell mixtures for 4 hours, the cells are pelleted by centrifugation at 200g for 5 minutes. The amount of ^{51}Cr released into the supernatant is measured by a gamma counter. The percent cytotoxicity is measured as cpm

in the test sample minus spontaneously released cpm divided by the total detergent released cpm minus spontaneously released cpm.

In order to block the MHC class I cascade a concentrated hybridoma supernatant derived from K-44 hybridoma cells (an anti-MHC class I hybridoma) is added to the test samples to a final concentration of 12.5% (Heike *et al.*, 1994, J. Immunotherapy 15:165-174).

An alternative to the chromium-release assay is the ELISPOT assay which measures cytokine release by cytotoxic T cells in vitro after stimulation with specific antigen. Cytokine release is detected by antibodies which are specific for a particular cytokine, such as interleukin-2, tumor necrosis factor α or interferon-γ (for example, see Scheibenbogen *et al.*, 1997, Int. J. Cancer, 71:932-936). The assay is carried out in a microtiter plate which has been pre-coated with an antibody specific for a cytokine of interest which captures the cytokine secreted by T cells. After incubation of T cells for 24-48 hours in the coated wells, the cytotoxic T cells are removed and replaced with a second labeled antibody that recognizes a different epitope on the cytokine. After extensive washing to remove unbound antibody, an enzyme substrate which produces a colored reaction product is added to the plate. The number of cytokine-producing cells is counted under a microscope. This method has the advantages of short assay time, and sensitivity without the need of a large number of cytotoxic T cells.

5.3.5 MONITORING OF EFFECTS DURING IMMUNOTHERAPY

The effect of immunotherapy with α2M polypeptide-antigenic molecule complexes can be monitored by any methods known to one skilled in the art, including but not limited to measuring: a) delayed hypersensitivity as an assessment of cellular immunity; b) activity of cytolytic T-lymphocytes *in vitro*; c) levels of infective agent-agent or tumor-specific antigens, *e.g.*, carcinoembryonic (CEA) antigens. In the case of the use of α2M – antigenic molecule complexes for prevention or treatment of cancer, the effect can additionally be monitored by: d) changes in the morphology of tumors using techniques such as a computed tomographic (CT) scan; and e) changes in levels of putative biomarkers of risk for a particular cancer in individuals at high risk, and f) changes in the morphology of tumors using a sonogram.

5.3.5.1 DELAYED HYPERSENSITIVITY SKIN TEST

Delayed hypersensitivity skin tests are of great value in the overall immunocompetence and cellular immunity to an antigen. Inability to react to a battery of common skin antigens is termed anergy (Sato *et al.*, 1995, Clin. Immunol. Pathol. 74:35-43).

Proper technique of skin testing requires that the antigens be stored sterile at 4°C, protected from light and reconstituted shorted before use. A 25- or 27-gauge needle ensures intradermal, rather than subcutaneous, administration of antigen. Twenty-four and 48 hours after intradermal administration of the antigen, the largest dimensions of both erythema and induration are measured with a ruler. Hypoactivity to any given antigen or group of antigens is confirmed by testing with higher concentrations of antigen or, in ambiguous circumstances, by a repeat test with an intermediate test.

5.3.5.2 IN VITRO ACTIVATION OF CYTOTOXIC T CELLS

The activity of cytotoxic T-lymphocytes can be assessed *in vitro* using the following method. Eight x 10^6 peripheral blood-derived T lymphocytes isolated by the Ficoll-Hypaque centrifugation gradient technique, are restimulated with $4x10^4$ mitomycinC-treated tumor cells in 3ml RPMI medium containing 10% fetal calf serum. In some experiments, 33% secondary mixed lymphocyte culture supernatant or IL-2, is included in the culture medium as a source of T cell growth factors.

In order to measure the primary response of cytolytic T-lymphocytes after immunization, T cells are cultured without the stimulator tumor cells. In other experiments, T cells are restimulated with antigenically distinct cells. After six days, the cultures are tested for cytotoxicity in a 4 hour ^{51}Cr-release assay. The spontaneous ^{51}Cr-release of the targets should reach a level less than 20%. For the anti-MHC class I blocking activity, a tenfold concentrated supernatant of W6/32 hybridoma is added to the test at a final concentration of 12.5% (Heike M., *et al.*, *J. Immunotherapy* 15:165-174).

5.3.5.3 LEVELS OF TUMOR SPECIFIC ANTIGENS

Although it may not be possible to detect unique tumor antigens on all tumors, many tumors display antigens that distinguish them from normal cells. The monoclonal antibody reagents have permitted the isolation and biochemical characterization of the antigens and have been invaluable diagnostically for distinction of transformed from nontransformed cells and for definition of the cell lineage of transformed cells. The best-characterized human tumor-associated antigens are the oncofetal antigens. These antigens are expressed during embryogenesis, but are absent or very difficult to detect in normal adult tissue. The prototype antigen is carcinoembryonic antigen (CEA), a glycoprotein found on fetal gut and human colon cancer cells, but not on normal adult colon cells. Since CEA is shed from colon carcinoma cells and found in the serum, it was originally thought that the presence of this antigen in the serum could be used to screen patients for colon cancer. However, patients with other tumors, such as pancreatic and breast cancer, also have elevated serum

levels of CEA. Therefore, monitoring the fall and rise of CEA levels in cancer patients undergoing therapy has proven useful for predicting tumor progression and responses to treatment.

Several other oncofetal antigens have been useful for diagnosing and monitoring human tumors, *e.g.*, alpha-fetoprotein, an alpha-globulin normally secreted by fetal liver and yolk sac cells, is found in the serum of patients with liver and germinal cell tumors and can be used as a marker of disease status.

5.3.5.4 COMPUTED TOMOGRAPHIC (CT) SCAN

CT remains the choice of techniques for the accurate staging of cancers. CT has proved more sensitive and specific than any other imaging techniques for the detection of metastases. A sonogram remains an alternative choice of technique for the accurate staging of cancers.

5.3.5.5 MEASUREMENT OF PUTATIVE BIOMARKERS

The levels of a putative biomarker for risk of a specific cancer are measured to monitor the effect of α2M covalently or noncovalently bound to antigenic molecule complexes. For example, in individuals at enhanced risk for prostate cancer, serum prostate-specific antigen (PSA) is measured by the procedure described by Brawer et. al., 1992, J. Urol. 147:841-845, and Catalona *et al.*, 1993, JAMA 270:948-958; and in individuals at enhanced risk for breast cancer, 16-α-hydroxylation of estradiol is measured by the procedure described by Schneider *et al.*, 1982, Proc. Natl. Acad. Sci. ISA 79:3047-3051.

5.4 TARGET AUTOIMMUNE DISEASES

Autoimmune diseases that can be treated by the methods of the present invention include, but are not limited to, insulin dependent diabetes mellitus (*i.e.*, IDDM, or autoimmune diabetes), multiple sclerosis, systemic lupus erythematosus, Sjogren's syndrome, scleroderma, polymyositis, chronic active hepatitis, mixed connective tissue disease, primary biliary cirrhosis, pernicious anemia, autoimmune thyroiditis, idiopathic Addison's disease, vitiligo, gluten-sensitive enteropathy, Graves' disease, myasthenia gravis, autoimmune neutropenia, idiopathic thrombocytopenia purpura, rheumatoid arthritis, cirrhosis, pemphigus vulgaris, autoimmune infertility, Goodpasture's disease, bullous pemphigoid, discoid lupus, ulcerative colitis, and dense deposit disease. The diseases set forth above, as referred to herein, include those exhibited by animal models for such diseases, such as, for example non-obese diabetic (NOD) mice for IDDM and experimental autoimmune encephalomyelitis (EAE) mice for multiple sclerosis.

The methods of the present invention can be used to treat such autoimmune diseases by reducing or eliminating the immune response to the patient's own (self) tissue, or, alternatively, by reducing or eliminating a pre-existing autoimmune response directed at tissues or organs transplanted to replace self tissues or organs damaged by the autoimmune response.

5.5 TARGET INFECTIOUS DISEASES

The infectious diseases that can be treated or prevented using the methods and compositions of the present invention include those caused by intracellular pathogens such as viruses, bacteria, protozoans, and intracellular parasites. Viruses include, but are not limited to viral diseases such as those caused by hepatitis type B virus, parvoviruses, such as adeno-associated virus and cytomegalovirus, papovaviruses such as papilloma virus, polyoma viruses, and SV40, adenoviruses, herpes viruses such as herpes simplex type I (HSV-I), herpes simplex type II (HSV-II), and Epstein-Barr virus, poxviruses, such as variola (smallpox) and vaccinia virus, RNA viruses, including but not limited to human immunodeficiency virus type I (HIV-I), human immunodeficiency virus type II (HIV-II), human T-cell lymphotropic virus type I (HTLV-I), and human T-cell lymphotropic virus type II (HTLV-II); influenza virus, measles virus, rabies virus, Sendai virus, picornaviruses such as poliomyelitis virus, coxsackieviruses, rhinoviruses, reoviruses, togaviruses such as rubella virus (German measles) and Semliki forest virus, arboviruses, and hepatitis type A virus.

In another embodiment, bacterial infections can be treated or prevented such as, but not limited to disorders caused by pathogenic bacteria including, but not limited to, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium tetani*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromotis*, *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter* (Vibrio) *fetus* , *Campylobacter jejuni*, *Aeromonas hydrophila*, *Bacillus cereus*, *Edwardsiella tarda*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella typhiimurium*, *Salmonella typhii*, *Treponema pallidum*, *Treponema pertenue*, *Treponema carateneum*, *Borrelia vincentii*, *Borrelia burgdorferi*, *Leptospira icterohemorrhagiae*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Pneumocystis carinii*, *Francisella tularensis*, *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Mycoplasma spp.*, *Rickettsia prowazeki*, *Rickettsia tsutsugumushi*, *Chlamydia spp.*, and *Helicobacter pylori*.

In another preferred embodiment, the methods can be used to treat or prevent infections caused by pathogenic protozoans such as, but not limited to, *Entomoeba histolytica*, *Trichomonas tenas*, *Trichomonas hominis*, *Trichomonas vaginalis*, *Trypanosoma gambiense*, *Trypanosoma rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani*, *Leishmania tropica*, *Leishmania braziliensis*, *Pneumocystis pneumonia*, *Plasmodium vivax*, *Plasmodium falciparum*, and *Plasmodium malaria*.

5.6 TARGET PROLIFERATIVE CELL DISORDERS

With respect to specific proliferative and oncogenic disease associated with α2M-α2M activity, the diseases that can be treated or prevented by the methods of the present invention include, but are not limited to: human sarcomas and carcinomas, *e.g.*, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma; leukemias, *e.g.*, acute lymphocytic leukemia and acute myelocytic leukemia (myeloblastic, promyelocytic, myelomonocytic, monocytic and erythroleukemia); chronic leukemia (chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia); and polycythemia vera, lymphoma (Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and heavy chain disease.

Diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by inhibiting the α2M function, include but are not limited to degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds; for example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues, etc.

5.7 DOSAGE REGIMENS AND FORMULATION

Covalent or noncovalent complexes of α2M polypeptides and antigenic molecules of the invention may be formulated into pharmaceutical preparations for administration to mammals for treatment or prevention of infectious diseases or cancer at therapeutically effective doses for immunotherapy.

Selection of the preferred effective dose will be determined by a skilled artisan based upon considering several factors which will be known to one of ordinary skill in the art. Such factors include the particular form of α2M, and its pharmacokinetic parameters such as bioavailability, metabolism, half-life, *etc.*, which will have been established during the usual development procedures typically employed in obtaining regulatory approval for a pharmaceutical compound. Further factors in considering the dose include the condition or disease to be treated or the benefit to be achieved in a normal individual, the body mass of the patient, the route of administration, whether administration is acute or chronic, concomitant medications, and other factors well known to affect the efficacy of administered pharmaceutical agents. Thus the precise dosage should be decided according to the judgment of the practitioner and each patient's circumstances, *e.g.*, depending upon the condition and the immune status of the individual patient, according to standard clinical techniques.

Covalent or noncovalent complexes of α2M polypeptides and antigenic molecules of the invention may be formulated into pharmaceutical preparations for administration to mammals for treatment or prevention of infectious diseases or cancer. Drug solubility and the site of absorption are factors which should be considered when choosing the route of administration of a therapeutic agent.

α2M polypeptide-antigenic molecule complexes of the invention may optionally be administered with one or more adjuvants in order to enhance the immunological response. For example, depending on the host species, adjuvants which may be used include, but are not limited to: mineral salts or mineral gels such as aluminum hydroxide, aluminum phosphate, and calcium phosphate; surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, and dinitrophenol; immunostimulatory molecules, such as cytokines, saponins, muramyl dipeptides and tripeptide derivatives, CpG dinucleotides, CpG oligonucleotides, monophosphoryl Lipid A, and polyphosphazenes; particulate and microparticulate adjuvant, such as emulsions, liposomes, virosomes, cochleates; or an immune stimulating complex mucosal adjuvants, Freund's (complete and incomplete, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.).

α2M polypeptide-antigenic molecule complexes of the invention may be administered using any desired route of administration, including but not limited to, *e.g.*, subcutaneously, intravenously or intramuscularly, although intradermally or mucosally is preferred. Advantages of intradermal or mucosal administration include use of lower doses and rapid absorption, respectively. Mucosal routes of administration include, but are not limited to, oral, rectal and nasal administration. Preparations for mucosal administrations are suitable in various formulations as described below. The route of administration can be varied during a course of treatment.

The doses recited above are preferably given once weekly for a period of about 4-6 weeks, and the mode or site of administration is preferably varied with each administration. In a preferred example, subcutaneous administrations are given, with each site of administration varied sequentially. Thus, by way of example and not limitation, the first injection may be given subcutaneously on the left arm, the second on the right arm, the third on the left belly, the fourth on the right belly, the fifth on the left thigh, the sixth on the right thigh, etc. The same site may be repeated after a gap of one or more injections. Also, split injections may be given. Thus, for example, half the dose may be given in one site and the other half on an other site on the same day.

Alternatively, the mode of administration is sequentially varied, *e.g.*, weekly injections are given in sequence subcutaneously, intramuscularly, intravenously or intraperitoneally.

After 4-6 weeks, further injections are preferably given at two-week intervals over a period of time of one month. Later injections may be given monthly. The pace of later injections may be modified, depending upon the patient's clinical progress and responsiveness to the immunotherapy.

Compositions comprising covalent or noncovalent complexes formulated in a compatible pharmaceutical carrier may be prepared, packaged, and labeled for treatment of the indicated infectious disease or tumor. In preferred aspects, an amount of α2M polypeptide – antigenic molecule complex is administered to a human that is in the range of about 2 to 150 μg, preferably 2 to 50 μg, most preferably about 25 μg, given once weekly for about 4-6 weeks, intradermally with the site of administration varied sequentially.

If the complex is water-soluble, then it may be formulated in an appropriate buffer, for example, phosphate buffered saline or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents, then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol. Thus, the covalent or noncovalent complexes and their physiologically acceptable solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the

nose) or oral, buccal, parenteral, rectal administration or, in the case of tumors, directly injected into a solid tumor.

For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well-known in the art.

Preparations for oral administration may be suitably formulated to give controlled release of the complexes. Such compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the complexes may be conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the complexes and a suitable powder base such as lactose or starch.

The complexes may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The complexes may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the complexes may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the complexes may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

The complexes may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the covalent or noncovalent complexes. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

The invention also provides kits for carrying out the therapeutic regimens of the invention. Such kits comprise in one or more containers therapeutically or prophylactically effective amounts of the covalent or noncovalent α2M polypeptide – antigenic molecule complexes in pharmaceutically acceptable form. The α2M polypeptide – antigenic molecule complexes in a vial of a kit of the invention may be in the form of a pharmaceutically acceptable solution, e.g., in combination with sterile saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluid. Alternatively, the complex may be lyophilized or desiccated; in this instance, the kit optionally further comprises in a container a pharmaceutically acceptable solution (*e.g.*, saline, dextrose solution, etc.), preferably sterile, to reconstitute the complex to form a solution for injection purposes.

In another embodiment, a kit of the invention further comprises a needle or syringe, preferably packaged in sterile form, for injecting the complex, and/or a packaged alcohol pad. Instructions are optionally included for administration of α2M polypeptide – antigenic molecule complexes by a clinician or by the patient.

6. EXAMPLE: α2M ANTAGONIZES HSP-MEDIATED ANTIGEN PRESENTATION VIA THE α2M RECEPTOR

6.1 INTRODUCTION

The Example presented herein describes the successful identification of an interaction between gp96 and the α2M receptor present in macrophages and dendritic cells *in vivo*, and the blocking of this interaction by α2M. The experiments presented herein form the basis for

the compositions and therapeutic methods of the present invention which relate to the use of α2M polypeptide-antigenic molecule complexes as immunotherapeutic agents for treatment of immune disorders, proliferative disorders, and infectious diseases.

The Applicant of the present invention noted that certain observations were inconsistent with a "direct transfer" model of HSP-chaperoned peptide antigen presentation. First, the immunogenicity of HSP preparations is dependent on the presence of functional phagocytic cells but not B cells or other nonprofessional antigen-presenting cells (Udono and Srivastava, 1993, *supra*; Suto and Srivastava, 1995, *supra*), whereas free peptides can sensitize all cell types. Second, extremely small quantities of HSP-peptide complexes were effective in eliciting specific immunity, *i.e.*, gp96-chaperoned peptides are several hundred times as effective as free peptides in sensitizing macrophages for CTL recognition, suggesting the possibility of a specific uptake mechanism. Third, gp96-chaperoned peptides elicited an MHC I response that was not limited by the size of peptide. Finally, the processing of gp96-peptide complexes in macrophage was found to be sensitive to Brefeldin A (BFA), which blocks transport through the Golgi apparatus, suggesting that processing occurred through an intercellular mechanism. These observations led to the hypothesis that HSP-chaperoned peptides may be processed internally and re-presented by MHC class I molecules on the cell surfaces of macrophages (Suto and Srivastava, 1995, *supra*). There is also the hypothesis that the mannose receptor is used in the uptake of gp96 but no mechanism has been proposed for the non-glycosylated HSPs, such as HSP70 (Ciupitu *et al.*, 1998, J. Exp. Med., 187: 685-691). Others suggested that a novel intracellular trafficking pathway may be involved for the transport of peptides from the extracellular medium into the lumen of ER) Day *et al.*, 1997, Proc. Natl. Acad. Sci. 94:8065-8069; Nicchitta, 1998, Curr. Opin. in Immunol. 10:103-109). Further suggestions include the involvement of phagocytes which (a) possess an ill-defined pathway to shunt protein from the phagosome into thecytosol where it would enter the normal class I pathway; (b) digest ingested material in lysosomes and regurgitate peptides for loading on the surface to class I molecules (Bevan, 1995, J. Exp. Med. 192:639-41). The discovery of a receptor for heat shock protein as disclosed herein helps to resolve the paradox of how extracellular antigenic peptides complexed to HSPs can be presented by MHC class I molecules on antigen presenting cells.

6.2 MATERIALS AND METHODS

Affinity chromatography. Proteins (1mg) in 2ml volume were incubated with 2ml of equilibrated AminoLink beads in PBS with a reductant ($NaCNBH_3$) for 1 hour. Uncoupled protein was removed by extensive washing of the column and unreactive groups quenched. Immobilization yields were typically >92% of the starting amount of protein. Columns were

stored at 4°C until used. Such columns were made with gp96 (purified as described in Srivastava *et al.*, 1986, Proc. Natl. Acad. Sci., U.S.A. 83:3407-3411) and albumin. For membrane purification, cells were lysed by dounce homogenization in hypotonic buffer containing PMSF. Unlyzed cells and nuclei were removed by centrifugation at 1000g for 5 mm. The postnuclear supematant was centrifuged at 100,000g for 90 mins. The pellet contains total membranes and was fractionated by aqueous two-phase partition with a dextran/polyethylene glycol biphase. Briefly membranes were resuspended in PEG (33% wt/wt in 0.22 M sodium phosphate buffer, pH 6.5) and underlaid gently with dextran (20%wt/wt in 0.22M sodium phosphate buffer, pH 6.5). The two phases were mixed gently and centrifuged at 2000 g for 15 mins. The white material at the interphase was enriched for plasma membranes, whose proteins were extracted by 2 hr incubation in 20mM Tris buffer (pH8, containing 0.08% octylglucoside) at 4°C.

Photo cross-linking of gp96 to putative receptor. The cross-linker (SASD, (Pierce) was labeled with I^{125} using iodobeads (Pierce). Radiolabeled SASD was covalently attached to gp96 by incubation at room temperature for 1 hr. Free SASD and I^{125} were removed by size exclusion column (KwikSep columns, Pierce). For cross-linking studies, I^{125}-SASD-gp96 (50 μg gp96) was incubated with purified $CD11b^{+}$ cells. Unbound protein was removed by washing. All procedures to this point were carried out in very dim light. Proteins were cross-linked with UV light. Cells were lysed with lysis buffer (0.5%NP4O, 10mM Tris, 1mMEDTA, 150mM NaCl) and treated with 100 mM 2-mercaptoethanol to cleave the cross-linker. *Cell lysates were analyzed by SDS-PAGE and autoradiography.*

Re-presentation assays. Re-presentation assays were carried out as described (Suto and Srivastava, 1995, Science 269:1585-1588). Antigen presenting cells (RAW264.7 macrophage cell line) were plated at a 1:1 ratio with AH I -specific T cells in complete RPMI. Approximately 10,000 cells of each type were used. Gp96 (10 μg/ml) chaperoning the AH1-20 mer peptide (RVTYHSPSYVYHQFERRAK) was added to the cells and the entire culture was incubated for 20 hrs. Stimulation of T cells was measured by quantifying the amount of IFN-γ released into the supernatants by ELISA (Endogen).

Protein Microsequencing. Proteins identified by affinity chromatography were analyzed on SDS-PAGE and stained with coomasie blue or transferred onto PVDF membrane and stained with coomasie blue, all of it under keratin-free conditions. Protein bands were excised with a razor from the gel or membrane. Tryptic peptides from an 80kDa faint coomassie band were extracted by 50% acetonitrile, 5% formic acid, dried, and loaded onto a 75 m 10 cm, reverse-phase C18, microcapillary column (3 μl vol) and tryptic peptides were separated by on-line microcapillary liquid chromatography-tendem mass spectrometry followed by database searching using the SEQUEST program as previously described.

(Gatlin *et al.*,2000, Anal. Chem. 72:757-63; Link *et al.*, 1999, Nat. Biotechnol. 17:676-82). The analysis was carried out in a data-dependent auto-MS/MS fashion using a Finnigan LCQ iontrap Mass Spectrometer.

6.3 RESULTS

Identification of an 80 kDa protein as a potential gp96 receptor. Homogenous preparations of gp96 were coupled to FITC and the gp96-FITC was used to stain RAW264.7 cells, shown to be functionally capable of re-presenting gp96-chaperoned peptides. Gp96-FITC but not control albumin-FITC preparations stained the cell surface of RAW264.7 cells (FIG. 1A). Plasma membrane preparations of cell surface-biotinylated RAW264.7 cells were solubilized in 0.08% octyl-glucoside and the soluble extract was applied to a gp96-Sepharose column. The bound proteins were eluted with 3M sodium chloride. SDS-PAGE analysis of the eluate showed 2 major bands of ~75-80 kDa size (FIG. 1B, top left). Blotting of this gel with avidin-peroxidase showed that both bands were biotinylated, indicating their surface localization (FIG. 1B, bottom left). Affinity purification of membrane extracts of RAW264.7 cells over control serum albumin affinity columns did not result in isolation of any proteins, nor did probing of immunoblots of such gels with avidin peroxidase detect any albumin-binding surface proteins (FIG. 1B, top and bottom center lanes). As an additional control, chromatography of membrane extracts of P815 cells which do not bind gp96-FITC and which do not re-present gp96-chaperoned peptides, on gp96 affinity columns did not result in elution of any gp96-binding proteins (FIG. 1B, top and bottom right lanes).

In parallel experiments, gp96 molecules were coupled to the radio-iodinated linker sulfosuccinimidyl (4-azidosalicylamido) hexanoate (SASD) which contains a photo cross-linkable group. Gp96-SASD-I^{125} was pulsed onto peritoneal macrophages, which have been shown previously to re-present gp96-chaperoned peptides (Suto and Srivastava, 1995, Science 269:1585-1588). Excess gp96-SASD was removed by multiple rounds of washing of the cells and photoactivation was carried out by exposure of cells to UV light for 10 mm. Cell lysates were reduced in order to transfer the I^{125} group to the putative gp96 ligand and were analyzed by SDS- PAGE followed by autoradiography. The gp96 molecule was observed to cross-link to an ~80 kDa band specifically present in re-presentation-competent macrophage but not in the re-presentation-incompetent P815 cells (FIG. 1C). This band appears to correspond in size to the larger of the two bands seen in eluates of gp96 affinity columns (FIG. 1D). No band corresponding to the lower band in that preparation is seen in the photo cross-linked preparation. The observation of a specific binding of gp96 to an 80 kDa protein in two different re-presentation-competent cell types, but not in a re-

presentation-incompetent cell line, and by two independent assays supported the candidacy of the 80 kDa molecule for the gp96 receptor.

Antiserum against the 80 kDa protein inhibits re-presentation of a gp96-chaperoned antigenic peptide. The eluates containing the 75-80 kDa proteins were used to immunize a New Zealand white rabbit, and pre-immune and immune sera were used to probe blots of plasma membrane extracts of the re-presentation-competent RAW264.7 and primary peritoneal macrophages and the re-presentation-incompetent P815 cells. The immune but not the pre-immune serum detected the 80 kDa band (and a faint lower 75 kDa band) in plasma membrane extracts of primary macrophage and the RAW264.7 membranes but not of P815 cells (FIG. 2A). The pre-immune and immune sera were tested in a functional assay for their ability to block re-presentation of gp96-chaperoned peptides. The L^d-restricted epitope AH1 derived from the gp70 antigen of murine colon carcinoma CT26 (Huang *et al.*, 1996, Proc. Natl. Acad. Sci. U.S.A. 93:9730-9735) was used as the model system. Complexes of gp96 with an AH1 precursor (used to inhibit direct presentation) were pulsed onto RAW264.7 cells which were used to stimulate a L^d/AH1-specific CD8+ T cell clone. Release of interferon-γ by the clones was measured as a marker of their activation. RAW264.7 cells were able to re-present gp96-chaperoned AH1 precursor effectively in this assay. It was observed that at the highest concentration, the immune sera inhibited re-presentation completely (FIG. 2B). Although the pre-immune serum was ineffective in inhibiting re-presentation as compared to the immune sera, it did inhibit re-presentation significantly at higher concentrations. The significance of this observation became clear later when we determined the identity of the gp96 receptor. Repeated immunizations with the affinity-purified gp96-binding proteins did not result in corresponding increase in antibody titers.

Identification of the 80 kDa protein as an amino terminal fragment of the heavy chain of the α2M receptor. The 80 kDa protein eluted from the gp96 affinity column was resolved on SDS-PAGE and visualized by staining with Coomassie Brilliant Blue. The protein band was subjected to in-gel trypsin digestion and mass spectrometry-based protein microsequencing as described in the methods in Section 6.2. Four independent tryptic peptides corresponding to N-terminal region of the α2-macroglobulin (α2M) receptor, designated by immunologists as CD91, were identified (FIG. 3C).

α2M inhibits re-presentation of a gp96-chaperoned antigenic peptide by RAW264.7. α2M receptor is one of the known natural ligands for the α2M receptor. Its ability to inhibit re-presentation of gp96-chaperoned antigenic peptide AH1 was tested in the assay described in FIG. 2. α2M but not control proteins selectin (CD62) or serum albumin was observed to inhibit re-presentation completely and titratably (FIG. 4). This observation was also consistent with the result in FIG. 2 that while the pre-immune serum did not detect an 80

kDa band in plasma membranes of RAW264.7 cells, it did inhibit re-presentation to some degree at high concentrations. Thus, by structural as well as functional criteria, the α2M receptor was determined to fulfill the criteria essential for a receptor for gp96.

6.4 DISCUSSION

The α2M receptor, which is also designated CD91, was initially identified as a protein related to the low density lipoprotein (LDL) receptor Related Protein (LRP) (Strickland *et al.*, 1990, J. Biol. Chem.265:17401-17404; Kristensen *et al.*, 1990, FEBS Lett. 276:151-155). The protein consists of an ~420 kDa α subunit, an 85 kDa β subunit and a 39 kDa tightly associated molecule (RAP). The α and β subunits are encoded by a single transcript of ~15 Kb in size (Van Leuven *et al.*, 1993, Biochim. Biophys. Acta. 1173:71-74. The receptor has been shown to be present in cells of the monocytic lineage and in hepatocytes, fibroblasts and keratinocytes. CD91 has been shown previously to bind the activated form of the plasma glycoprotein α2M, which binds to and inhibits a wide variety of endoproteinases. α2M receptor also binds to other ligands such as transforming growth factor β (O Connor-McCourt *et al.*, 1987, J. Biol. Chem. 262:14090-14099), platelet-derived growth factor (Huang *et al.*, 1984, Proc. Natl. Acad. Sci. U.S.A. 81:342-346), and fibroblast growth factor (Dennis *et al.*, 1989, J. Biol. Chem. 264:7210-7216). α2M is thus believed to regulate, and specifically diminish, the activities of its various ligands. Complexed with these various ligands, α2M binds α2M receptor on the cell surface and is internalized through receptor-mediated endocytosis. Uptake of α2M-complexed ligands has been assumed thus far to be the primary function of the α2M receptor, although a role for it in lipid metabolism is also assumed. α2M receptor ligands other than α2M, such as tissue-specific plasminogen activator-inhibitor complex (Orth *et al.*, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:7422-7426) and urokinase-PAI1 complex (Nykjaer *et al.*, 1992, J. Biol. Chem. 267:14543-14546), have been identified. These ligands attest to a role for α2M receptor in clearing a range of extracellular, plasma products.

The studies reported here show that the heat shock protein gp96 is an additional ligand for the α2M receptor. The human gp96-coding gene has been mapped previously by us at chromosome 12 (q24.2→q24.3) (Maki *et al.*, 1993, Somatic Cell Mol. Gen. 19:73-81). It is of interest in this regard that the α2M receptor gene has been mapped to the same chromosome and at a not too distant location (q13→q14) (Hilliker *et al.* Genomics 13:472-474). Gp96 binds α2M receptor directly and not through other ligands such as α2M. Homogenous preparations of gp96, in solution, or cross-linked to a solid matrix, bind to the α2M receptor. Indeed, the major ligand for the α2M receptor, α2M, actually inhibits interaction of gp96 with α2M receptor, instead of promoting it, providing evidence that gp96

is a direct ligand for the α2M receptor. The 80 kDa protein shown to bind gp96 is clearly an amino terminal degradation product of the α subunit of the α2M receptor. Degradation products of the α2M receptor in this size range have also been observed in previous studies (Jensen *et al.*, 1989, Biochem. Arch. 5:171-176), and may indicate the existence of a discrete ectodomain in the α2M receptor which may be particularly sensitive to proteolytic cleavage.

As shown here, the gp96- α2M receptor interaction provides a new type of function for α2M receptor, a function of a sensor, not only of the extracellular environment with its previously known plasma-based ligands, but also a sensor of the intracellular milieu as well. HSPs such as gp96 are obligate intracellular molecules and are released into the extracellular milieu only under conditions of necrotic (but not apoptotic) cell death. Thus, the α2M receptor may act as a sensor for necrotic cell death (see FIG. 5), just as the scavenger receptor CD36 and the recently identified phosphatidyl serine-binding protein act as sensors of apoptotic cell death and receptors for apoptotic cells (Savill *et al.*, 1992, J. Clin. Invest.90:1513-1522; Fadok *et al.*, 2000, Nature 405:85-90). Interaction of the macrophages with the apoptotic cells leads to a down-regulation of the inflammatory cytokines such as TNF (Fadok *et al.*, 2000, *supra*), while gp96-APC interaction leads to re-presentation of gp96-chaperoned peptides by MHC I molecules of the APC, followed by stimulation of antigen-specific T cells (Suto and Srivastava, 1995, *supra*) and, in addition, secretion of pro-inflammatory cytokines such as TNF, GM-CSF and IL-12. Interestingly, α2M, an independent ligand for the α2M receptor, inhibits representation of gp96-chaperoned peptides by macrophages. This observation suggests that re-presentation of gp96-chaperoned peptides can not occur physiologically in blood, but only within tissues as a result of localized necrotic cell death. This is consistent with the complete absence of gp96 or other HSPs in blood under all conditions tested. Together, these observations point towards a possible mechanism whereby the release of HSPs in the blood as a result of severe tissue injury and lysis will not lead to a systemic and lethal pro-inflammatory cytokine cascade.

It is possible, therefore, that the α2M receptor renders it possible for the APCs to sample (i) the extracellular milieu of the blood through α2M and other plasma ligands and (ii) the intracellular milieu of the tissues through HSPs, particularly of the gp96 family. The former permits APCs to implement their primordial phagocytic function, while the latter allows them to execute its innate and adaptive immunological functions. Viewed in another perspective, recognition of apoptotic cells by APCs through CD36 or phophatidyl serine, leads to anti-inflammatory signals, while interaction of the APC with necrotic cells through α2M receptor leads to pro-inflammatory innate and adaptive immune responses (see Srivastava *et al.*, 1998, Immunity 8: 657-665).

The invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references cited herein, including patent applications, patents, and other publications, are incorporated by reference herein in their entireties for all purposes.